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Defining the Doratopsis: Investigating the Endpoint of the Paralarval Stage in Chiroteuthis Calyx

Alicia Bitondo
California State University, Monterey Bay

DEFINING THE DORATOPSIS: Investigating the endpoint of the paralarval stage in *Chiroteuthis calyx*

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in

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by

Alicia Bitondo

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CALIFORNIA STATE UNIVERSITY MONTEREY BAY

The Undersigned Faculty Committee Approves the

Thesis of Alicia Nicole Bitondo:

DEFINING THE DORATOPSIS: Investigating the endpoint of the paralarval stage in

Chiroteuthis calyx

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Jonathan Geller, Chair
Moss Landing Marine Labs

_____

Scott Hamilton
Moss Landing Marine Laboratories

_____

Kenneth Coale
Moss Landing Marine Laboratories

Digitally signed by Kris Roney
Date: 2016.12.21 14:15:48 -08'00'

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Kris Roney, Dean
Associate VP for Academic Programs and Dean of Undergraduate and Graduate Studies

DEDICATION

This work is dedicated to my family: Liz, Vinny, Suzanne, Amy, Jeff, Adam, and little Annalisa. You guys are my world and I couldn't have done this without your constant support and love. Thank you.

“What would the ocean be without a monster lurking in its depths? It would be like sleep without dreams.”

-Werner Herzog

ABSTRACT

Defining the doratopsis: Investigating the endpoint of the paralarval stage in *Chiroteuthis calyx*
by
Alicia Nicole Bitondo
Master of Science in Marine Science
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The life histories of open ocean squid are often poorly understood, due to the difficulty of collecting and observing them in the wild. In order to understand life histories fully, species must be identifiable at all life stages. In cephalopods there is uncertainty surrounding the endpoint of the paralarval stage, and this boundary must consequently be determined on a species by species basis. Some common morphological events used as markers of this life stage change are the loss of paralarval characters, and rapid changes in the relative size and growth of certain key structures. Alternatively, the end of the paralarval stage is sometimes defined ecologically, by changes in predatory behavior, diet, and distribution.

In this study I take a comprehensive survey of all possible markers of the end of the paralarval stage in *Chiroteuthis calyx*, the Swordtail Squid. This squid is abundant in the mesopelagic community of Monterey Bay, California, and is significant in the diet of local predators. Its paralarvae, like those of other chiroteuthids, are known as "doratopses," readily identified by their long and ornate tails. Live specimens were collected via Remotely Operated Vehicle and Tucker Trawl from the Monterey Bay Submarine Canyon. Preserved specimens were obtained from the Monterey Bay Aquarium Research Institute's (MBARI) collection. Additionally, animals captured

within the Video and Annotation Referencing System (VARS) at MBARI were analyzed and equations were derived to estimate their mass and tail length.

This species has three paralarval characters, the brachial pillar, tail, and paralarval club, all of which are lost at some point during the transition from doratopsis to sub-adult. The presence and absence of these three characters were plotted in a logistic regression, and a non-metric Multidimensional Scaling analysis. The loss of the three characters were found to be simultaneous, meaning that the historical morphological marker of the doratopsis, the tail, is an appropriate signifier of the end of the paralarval stage. The loss of the paralarval club and subsequent development of the adult tentacle club has a significant effect on predatory behavior, as the adult clubs are equipped with photophore lures for catching fish. The length of the tail is observed to be variable amongst paralarvae, and length was examined with respect to mass to look for trends in size. It was found that tail length does not correlate with mass, and so tail length is not an appropriate tool with which to estimate size or age.

Relative growth of key structures was examined using an Analysis of Covariance (ANCOVA) between life stages (as determined by presence of the paralarval club), with Dorsal Mantle Length (mm) as the independent variable. These structures included those associated with body shape, the feeding apparatus, and locomotion. The results indicated consistent changes in the size and relative growth of the feeding apparatus and body shape during the metamorphic transition between paralarvae and sub-adult. The fin length and shape did not exhibit significant changes during metamorphosis, but the fin width did enlarge relative to the body size in sub-adults. These results indicate a potential change in habitat, activity level, and predator-prey interactions following a shift

from a long and cylindrical morphology (with a tail mimicking a common siphonophore) to a wider and more spherical body shape. The reduction of hydrodynamic efficiency and changes in tentacle morphology indicates a shift to a more sedentary lifestyle and a new mode of feeding.

The adults have been observed eating myctophid fish, but the diet of the paralarvae is unknown. To examine dietary change, pigmentation of the digestive gland was used for a qualitative comparison. Again life stage was designated according to presence of the paralarval club. The majority of paralarvae had red digestive glands, while sub-adults had a majority of orange or yellow digestive glands, suggesting a change in diet from pigment-rich zooplankton to fish which was associated with morphological changes consistent with shifts in prey capture techniques.

Trends in depth distribution were examined with both a gradual ontogenetic descent, and diel vertical migration (DVM) in mind. A VARS query for all *Chiroteuthis calyx* observed from 1998-2013 revealed a significant difference in mean depth between life stages. To examine differences in variability that would indicate vertical mobility in the water column, depth *vs* mass for specimens in this study were examined and found to not only have a difference in mean depth, but much higher variability in the paralarvae indicated that the behavior of DVM was present in the paralarvae but not the sub-adults.

The results of this study reveal a coordinated process of metamorphosis involving morphological and ecological factors. The difference between paralarvae and adults is therefore drastic both in their appearance and their behavior, suggesting that perhaps the typical description of squid development as gradual is not always true.

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INTRODUCTION

BACKGROUND

A complete understanding of population dynamics and life history requires that species be identifiable at all life stages (Ramos-Castillejos et al. 2010). In many cases this is complicated by large ecological and morphological differences between hatchlings and adults. The process of metamorphosis involves morphological and ecological changes that turn a hatchling larva into a juvenile or sub-adult. This process is seen in invertebrates and vertebrates, but is particularly rapid in most marine invertebrates, as they radically transform from free swimming larvae into sessile juveniles on the benthos (Pechenik, Wendt, and Jarrett 1998). The rapid nature of this process is necessary for a complete niche change to be possible.

Cephalopods are different from most marine invertebrates as many develop gradually and directly (Elizabeth K. Shea and Vecchione 2010). The term paralarva is used in cephalopods rather than larva for this reason (Vidal 2014). However, somatic growth that spans orders of magnitude in size and weight may require significant behavioral changes, such as in diet and habitat, suggesting that in some cases, a rapid and comprehensive metamorphosis may be necessary (Pechenik *et al*., 1998; Hadfield 2000; Hadfield *et al*., 2001; Almansa *et al*., 2006). Shea and Vecchione (2010) define paralarvae as "newly hatched cephalopods that have a distinctly different mode of life than the adults, with an endpoint identified by ecologically significant allometric changes in morphological characters."

The greatest diversity and biomass of cephalopods is found in the open ocean, and the majority of these are squid (Boyle and Rodhouse 2005). Despite their abundance and ecological significance, little is known about the life histories of these pelagic squid (Almansa *et al*., 2006; Caddy and Rodhouse, 1998; Piatkowski *et al*., 2001; Staudinger *et al*., 2013). Data describing their ecology is challenging to collect for a number of reason, including the vast expanse and often inaccessibility of their environment, the agility that makes them adept at escaping collection, the complexity of their life cycles, their delicate tissues, and the difficulty of keeping and rearing them in the laboratory (Vecchione *et al*., 1991; Piatkowski *et al*., 2001; Zeidberg 2004).

In the absence of ecological data, morphological changes are relied upon as a way to distinguish between the paralarval and sub-adult life stages. The diversity of morphology and ecology in cephalopods requires that the metamorphic shift from paralarva to juvenile be defined on a "species by species basis," (Elizabeth K. Shea and Vecchione 2010). Often there is a morphological "marker" signifying the transition, the appearance or disappearance of which is predicted to coincide with ecological changes such as in diet or habitat (Elizabeth K. Shea and Vecchione 2010).

Among the more commonly studied pelagic squid are the Chiroteuthidae, gelatinous squid present in mesopelagic (200-1000 m) oceans worldwide (Franco-Santos and Vidal 2014). Their unifying characteristic is a paralarval stage known as a doratopsis. Doratopses are readily identified by a long, ornate tail (absent in the adult stages of all but one species), and an elongate neck and brachial pillar (Vecchione *et al*., 1991; Nixon and Young 2003; Young *et al*., 2008; Franco-Santos and Vidal 2014). Though color and shape varies interspecifically, the tail is always large relative to the body length

(Vecchione *et al*., 1991; Nixon and Young 2003). Indeed, doratopses of the Atlantic species *Chiroteuthis veranyi* appear so distinct from adults that they were initially misidentified as a separate species altogether- *Doratopsis vermicularis*, a now defunct taxon whose name was appropriated to the unique paralarval stage (Young, 1991; Vecchione *et al*., 1992).

Chiroteuthis calyx, the Swordtail squid, is the only chiroteuthid reported in the waters off California, and its abundance makes it a significant dietary component of large local predators such as the blue shark (Vecchione *et al*., 1991; Staudinger *et al*., 2013). The adult form is muscular, spherical in shape, with large eyes and a wide head, while the paralarva has a cylindrical body, with the name-giving tail exhibiting bright orange pouches along its length (Young 1991; Vecchione *et al*., 1991; Bush *et al*., 2009; Figure 1). The dramatic changes in body plan that occur between the paralarval and adult stages in this species pose a unique opportunity to examine the morphological changes that occur during metamorphosis and the factors triggering the metamorphic transition in a representative midwater squid.

A) B)

Figure 1: *Chiroteuthis calyx* life stages A) Paralarva B) Sub-adult

The loss of the tail has historically represented the boundary between paralarvae and juveniles in the Chiroteuthidae, as it is the most conspicuous dimorphism between paralarvae and sub-adults (Vecchione *et al*., 1991; Nixon and Young 2003). In some individuals, the tail is found to be as long or longer than the mantle, comprising a huge portion of the animals' length, and affecting the hydrodynamic profile (Vecchione *et al*., 1991). Tail size appears to vary among individuals (Bitondo, pers. obs.), and Vecchione et al. (1991) postulate that the tails might be gradually resorbed. It is also possible that the variation in tail lengths is due to mechanical damage. However, when bent during collection, specimens' tails always break at the proximal end (Vecchione, Robison, and Roper 1991). If tail loss is due to mechanical damage this event may not correlate closely to other more significant ontological changes, and the boundary between paralarva and sub-adult should be redefined.

PARALARVAL STRUCTURES

Metamorphic changes from paralarval to sub-adult squid often involve the loss of paralarva-specific structures (Vidal 2014). In addition to the tail, there are two other paralarval structures in this family: the brachial pillar and the paralarval tentacle club (Vecchione *et al*., 1991; Sweeney *et al*., 1992; Voss *et al*., 1998; Nixon and Young 2003; Shea and Vecchione 2010; Figure 2).

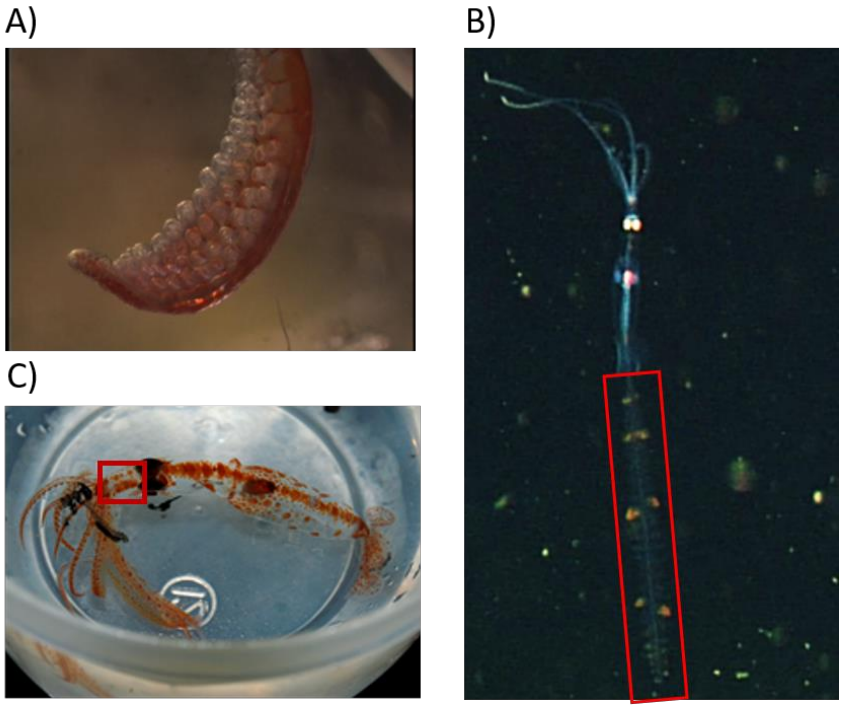


Figure 2: Paralarval structures examined in *Chiroteuthis calyx*. A) Paralarval club B) Tail, and C) Brachial Pillar

The brachial pillar is a vesiculate section of flesh between the buccal mass and the brachial crown. It can be filled with ammonium to adjust buoyancy, and also serves to elongate the appearance of the individual. The development of the tentacle clubs of chiroteuthid doratopses is outlined by Roper and Young (1999). There is a paralarval club, with unstalked, muscular suckers in four rows, and the adult club suckers develop along the tentacle stalk (Mensch 2010; Nixon and Young 2003). During metamorphosis, the paralarval club is resorbed in a process unique among modern cephalopods (Young, 1991). All 3 larval characters (Figure 2) can potentially serve as distinguishing markers between life stages, but it is not known how they correlate to other morphological and ecological changes.

RELATIVE GROWTH RATES

Growth curves are a useful tool to investigate the timing of developmental milestones, as inflection points warranting the assignment of break points in a growth curve signify life stage change (Rodhouse and Nigmatullin 1996; Elizabeth K. Shea and Vecchione 2010). In some cases there are more than one break point in the growth of a single structure, and break points are not always simultaneous among different structures. Growth of a specific body part with respect to the whole animal is represented by the allometric function, , $y=kx^b$ (E. K. Shea and Vecchione 2002). "k" and "b" are both constants, while x is the independent variable. For squid, the Dorsal Mantle Length (DML) is often used as "x", an approximation of whole animal size (Boyle and Rodhouse 2005).

Accelerated growth after a change in life stage of any animal is most often seen in the feeding and locomotory structures, although changes in allometry are also not necessarily correlated with behavioral changes (Uchikawa and Kidokoro 2014; Vidal 2014). The shape of the body and the size of the fins affect the locomotive efficiency of the animal, and are indicators of activity level. The functional mouth of a squid includes its appendages-- collectively known as the brachial crown-- in addition to the beak and buccal mass. The size of the brachial crown and beak are directly related to the maximum size of prey (Rodhouse and Nigmatullin 1996; Uchikawa and Kidokoro 2014). The rostrum is the main protrusion of the beak, and a longer rostrum is able to pierce flesh more deeply. The upper and lower rostral and jaw angles form the "biting surfaces" (Uyeno and Kier 2007).

DIET

Observations via Remotely Operated Vehicle (ROV) indicate that adult *C. calyx* feed on myctophid fish. While younger squid usually feed upon small crustaceans, then bigger crustaceans, and finally fish and cephalopods, the exact diet of *C. calyx* paralarvae remains unknown (Boyle and Rodhouse 2005; Rodhouse and Nigmatullin 1996). Dietary analyses in deep sea squid are complicated by a number of variables. The squid's digestion and absorption are quick (as little as 3-6 hours in active swimmers), they have a tendency towards net feeding during trawls, and they exhibit "selective rejection" when encountering large or difficult to swallow food items, meaning that the hard identifiable parts of some prey items may not be ingested (Hanlon 1997; Rodhouse and Nigmatullin 1996).

Some compounds are absorbed and stored in the digestive gland, and so in the absence of gut content data, this structure may serve as an indicator of the animal's diet (Semmens 1998; Semmens 2002; Almansa *et al*., 2006; Boucher-Rodini and Boucaud-Camou 2012). If prey items contain pigments, the digestive gland will also contain these pigments. Therefore, changes in pigmentation may be useful as a way to qualitatively track diet changes from one trophic level (i.e. planktivorous) to the next (i.e. piscivorous).

DISTRIBUTION

The ocean's depths are divided into strata that confer specific challenges for survival (Robison *et al*., 1998; Robison 2004; Boyle and Rodhouse 2007). The upper 200 meters is known as the epipelagic or euphotic zone, where light and primary production are plentiful. The mesopelagic zone lies between 200 and 1000 meters,

spanning the transition from the brightly lit surface waters to the darker regions of the deep sea. Changes in depth distribution are common among pelagic squid, and can occur gradually or more rapidly.

Gradual ontogenetic descent is a common feature in the life history of mesopelagic squid, as they change from epiplanktonic to nektonic (Nixon and Young 2003; Rodhouse and Nigmatullin 1996). Increasing depth involves a reduction in light, temperature, pH, oxygen, and prey availability (Robison 2004). Mean daytime depth of *C. calyx* historically indicates an ontogenetic descent (Roper and Young 1975; Hunt 1996; Voss *et al*., 1998).

Diel vertical migration (DVM) is another common phenomenon in pelagic squid, representing a more rapid change in depth. Individuals remain deeper during the daytime, and travel up to shallower, more productive waters at night. Zooplankton often make this daily migration, and zooplankton predators often follow and track their prey. In many squid species, vertical migration is a behavior only exhibited in post-larval individuals, and the onset of vertical migration has been suggested as a divider between the paralarval and sub-adult life stages (Elizabeth K. Shea and Vecchione 2010). There are indications that *C. calyx* paralarvae and adults are vertical migrators, though these results are not conclusive (Voss *et al*., 1998). The behavior of Batesian mimicry has recently been confirmed in *C. calyx* doratopses, as they strongly resemble a venomous siphonophore, *Nanomia bijuga* (Burford *et al*., 2014). *Nanomia* is a vertical migrator, and to remain within the densest distribution of its model, the doratopses may also vertically migrate.

STUDY OBJECTIVES

I investigated five different changes surrounding the period of metamorphosis to determine their relative timing. The objective was first to find a morphological marker that could be used to designate the end of the paralarval stage, and then to compare that to other possible changes to see if metamorphosis is one simultaneous and coordinated process, or a series of morphological and ecological changes that may or may not be related.

Morphological traits examined were 1) the loss of the three paralarval characters as possible markers, 2) the change in tail length with size, and 3) the relative growth rates of key structures. It was expected that the tail loss will correlate with the loss of the other larval characters, but that tail length will not decrease leading up to metamorphosis. I also expected that the relative size of key structures will change in size and relative growth rate based on life stage.

Ecological changes were inferred from 1) the digestive gland color as an indication of diet change, and 2) the depth distribution by life stage and by mass to indicate ontogenetic descent and DVM. I expected that the paralarval digestive glands would be mostly red, while the digestive glands of sub-adults would show mostly yellow and orange pigmentation, indicating a decrease in the amount of zooplankton consumed. The depth distribution was examined first in a binary fashion to investigate a difference in mean daytime depth between paralarvae and sub-adults/adults. A difference here would indicate ontogenetic descent. Lastly, depth in paralarvae and sub-adults will be examined to look for changes in the variance of the depth distribution, with high variability as an indicator of DVM.

Each of these analyses may lead to a redefined boundary between the doratopsis and the sub-adult *C. calyx*. If they are synchronized, it will be evidence for a more rapid and comprehensive metamorphosis in a pelagic squid.

MATERIALS AND METHODS

MATERIALS EXAMINED

Ninety-two specimens of *Chiroteuthis calyx* were examined, including live and preserved animals. Sixty-two live specimens were collected from August 2012 to November 2014 via Remotely Operated Vehicle (ROV) and Tucker trawls in the Monterey Bay at sites offshore of Moss Landing in the Monterey Submarine Canyon.

ROV collection was done with the ROV *Doc Ricketts*, operated aboard the *R/V Western Flyer*, and the ROV *Ventana*, aboard the *R/V Rachel Carson*. ROV collection included detritus sampling and suction sampling. The detritus sampler was a clear cylinder with two moveable lids which was maneuvered over the animal to avoid direct contact. Eight specimens were captured with this method. Two of these had tails, both of which broke off at the time of capture for a 0% retention rate. The suction sampler consisted of a long hose that provided adjustable levels of light suction to pull the animal into a collection vessel. Thirty-one specimens were obtained with this method. Seventeen of these had tails, five of which were broken off at the time of capture for a 71% retention rate. Twenty-three animals were collected using a Tucker Trawl at 600m. These specimens were often the smallest collected, but were damaged and all tails were broken off for a 0% retention rate.

Collected animals were returned to the surface tender vessel where they were euthanized by one of two methods: 90% ethanol bath or freezing at -80oC (IACUC Reference # EX-07082015). Morphological measurements (Figure 3) on live animals were made pre-euthanasia. Preserved specimens were obtained from MBARI's midwater lab. This included 12 whole bodies and 8 heads frozen at -80°C, and 10 whole bodies preserved in formalin. None of the preserved samples had tails intact. All measurements were taken on the right side of the squid using a 15 cm ruler, to the nearest 0.5mm (Moltschaniwskyj 1995).

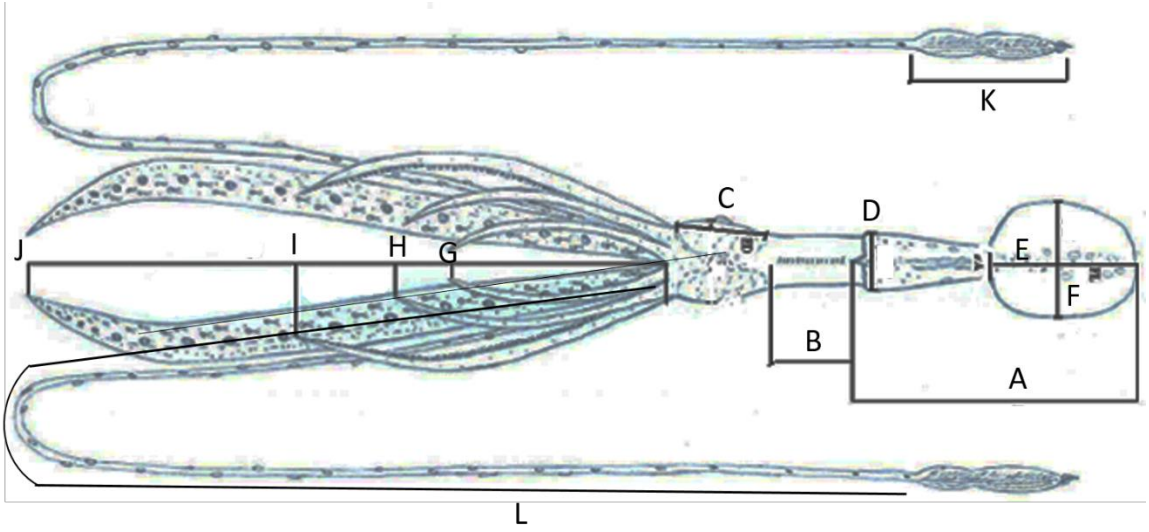


Figure 3: *Chiroteuthis calyx* external morphology. A)Dorsal Mantle Length (DML); B)Neck Length (NL); C)Eye Diameter (ED); E)Fin Length (FL); F)Fin Width (FW); G)Arm 1 (A1); H)Arm 2 (A2) ; I)Arm 3 (A3); J)Arm 4 (A4); K) Tentacle Club Length (TCL); L)Tentacle Length (TL). Modified with permission from (Mensch 2010).

The upper rostral length (URL) of the beak is the main piercing surface, and its relative size is indicative of prey size. Beaks were removed and preserved in 90% ethanol, and later photographed with a ruler with 0.5 mm increments for scale. URL was

determined using the program ImageJ (Abràmoff, Magalhães, and Ram 2004).

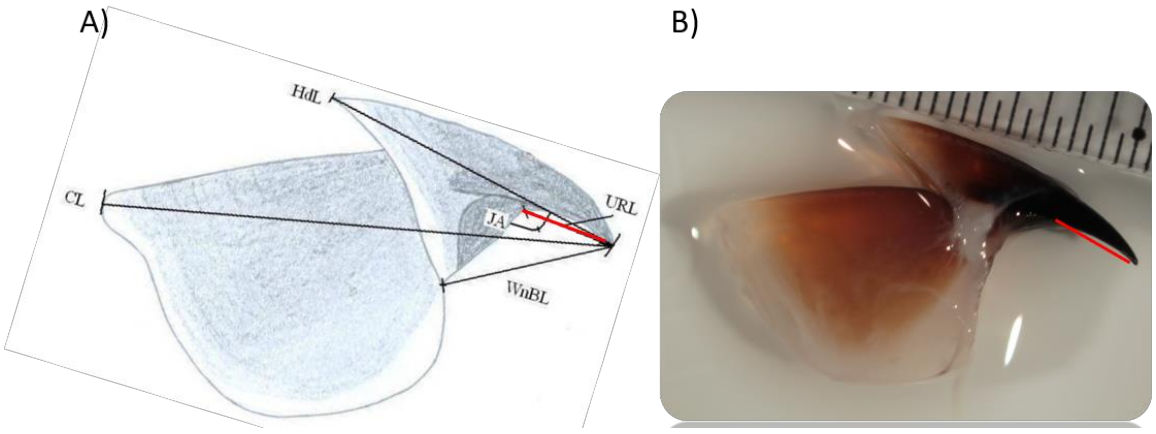


Figure 4: Upper Rostral Length (highlighted in red): A) Typical squid upper jaw, (adapted, with permission from Mensch 2010); B) *Chiroteuthis calyx* upper jaw, with 0.5mm ruler

Depth data was obtained for specimens collected with the ROV, and for preserved specimens for which depth had been recorded. Depth data on Tucker Trawl specimens was not specific enough to use reliably. Tail and depth data availability are summarized in Table 1.

Table 1: Sample sizes of *Chiroteuthis calyx* collected with intact tails and known depth information.

	Present (N)	**Absent (N)**	**Unknown (N)**
Tail	18	18	56
	Known (N)		**Unknown (N)**
Depth	43		48

SUPPLEMENTAL ANIMALS FROM THE VARS DATABASE

Ecological information on deep sea squid is often inferred from the morphology of dead specimens whose delicate tissues are easily damaged during collection (Hoving et al. 2013). The advent of *ROVs* in the last few decades made possible *in situ* observation of pelagic squid, adding immensely to our ability to study and understand their role in midwater ecology (Robison 2004). The Video Annotation and Reference System (VARS), operated by the Monterey Bay Aquarium Research Institute (MBARI), has thousands of hours of footage of deep sea animals in its archives.

To increase sample size of animals for which tail length (TLL) and depth could be calculated, 31 individual records were randomly selected from a query for all *C. calyx* seen in the VARS database. The selected individuals had sufficient footage so that clear photos, in which the animals were normal to the direction of view, could be captured and analyzed.

Equation 1: The DML of the animals in the photos could not be determined, as there were no known lengths to which the animal size could be compared. Instead, allometric relationships derived from intact physical specimens were used to derive an equation to approximate mass of the VARS specimens, after Zeidberg *et al*., (2004). The ratio of DML to ED is a robust proxy for mass from still images because these two parameters are relatively conspicuous, exist along the same plane, and the ratio changes as a function of mass. The relationship between mass, ED and DML for the specimens in this study is represented by Equation 1, with r^2=0.77. This formula was used to

approximate the mass of animals in the VARS photos. Still shots were obtained when the animal was in sharp focus and parallel to the camera.

$$\ln(mass) = 6.06 + 2.18 * \ln\left(\frac{ED}{DML}\right) \quad \text{(Equation 1)}$$

Equation 2: The relationship between tail length and number of pouch pairs was examined so that tail length of animals observed in the VARS database might be approximated (Figure 5). Equation 2 shows the relationship between Tail Length (TLL) and number of pouch pairs for specimens collected, with r^2=0.94:

$$TLL(mm) = 2.664 + 16.16(pouchpairs) + 3.19(pouchpairs)^2 \quad \text{(Equation 2)}$$

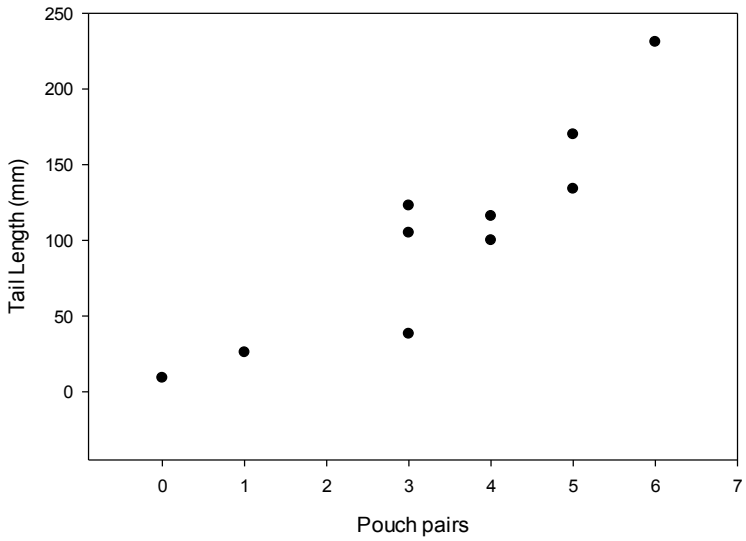


Figure 5: Tail length *vs* number of pouch pairs in paralarval *Chiroteuthis calyx*.

Equation 3: Wet weight mass was measured in grams for 48 physical specimens. The mass of 43 specimens could not be measured because they were either incomplete or preserved in a fixative that tends to de-water the fixed specimen. It was important to be able to estimate mass in order to increase sample size for the later comparisons of mass *vs* TLL and mass *vs* depth. ED and DML both showed a high correlation with mass

(r^2=0.86 for both), and these variables were selected to establish a formula to estimate mass from specimens where this quantity could not be obtained. (JMP®, Version *12.1.0*. SAS Institute Inc., Cary, NC, 1989-2007).

A stepwise regression was run using DML and ED to find the most appropriate model for this relationship. The Aikake information criterion for small sample sizes (AICc) was used to determine the best-fit model. The lower AICc is likely to be the better model, and an absolute difference of 2 or more is considered a significant difference (Anderson and Burnham 2002). When ED alone was used as a predictor, the AICc was 57.1. When DML alone was used, AICc was 76.7. When both variables were used, the AICc was the lowest, at 30.2, making this by far the best fit model. The relationship is represented by Equation 3, with r^2=0.97:

$$\mathrm{Ln}(mass) = (0.18 * ED) + (0.041 * DML) - 2.21 \qquad \text{(Equation 3)}$$

PARALARVAL STRUCTURES

In *C. calyx*, the maturation of the adult tentacle club is the most appropriate marker of life stage, as it is highly ecologically significant in enabling the animal to catch fish. The distal end of the adult club bears one photophore; these are dangled in a "fishing" posture to attract prey (Hunt 1996; Nixon and Young 2003; Robison 2004). The utilization of sucker hooks is also an indication of a dietary shift to fish (Hanlon 1997). Shea and Vecchione (2010) found that, for *Brachioteuthis*, changes in the arm and club sucker morphology was "reasonable" as a character to designate the boundary of metamorphosis. The development of the adult club was categorized into 4 stages, to

determine at what level of development it possessed all the characters of a mature adult club. This stage of development was designated as the end of the paralarval stage.

The presence and absence of the three paralarval characters (Figure 2) were examined to determine if the losses of these three structures were synchronized. First, logistic regressions of the presence and absence of all three characters were plotted against both DML and mass. If all three structures change simultaneously, it was predicted that the L_{50} and M_{50}, as measures of 50% structure loss, would overlap.

To measure the correlation of all three, a non-metric Multidimensional Scaling Analysis (nMDS) was performed analyzing presence and absence for all three characters. A distance matrix was created using Manhattan distance. That distance matrix was used to create the nMDS plot based on the similarities in pairwise distance estimates between samples. The closer two points are to each other, the more similar they are regarding the three traits. It was expected that those designated as paralarvae or sub-adults based on club morphology would be grouped closer to themselves than each other.

TAIL LENGTH

The ends of the tails of individual doratopses are highly variable, and the distal ends are always broken (Bitondo, pers. obs.). I expected that tail length would not correlate with body size. The length of the tails with respect to mass was examined to look for any trends that would indicate a shortening of the tail with increased body size. Twelve physical specimens had tails that could be directly measured. Tail length for six damaged specimens was approximated from video footage using the ratio of the tail to the DML *in situ*, with the DML measured directly on the captured animal. Additionally,

18 specimens from VARS were analyzed for mass and tail length using Equations 1 and 2, respectively. The length of the tail with respect to mass was then plotted, and a linear regression was performed to analyze the data for trends.

RELATIVE GROWTH RATES

In some cephalopods, the end of the paralarval stage is associated with radical changes in body proportions. Key structures are plotted against a measurement that approximates animal size, usually DML in squid. An abrupt change in the slope of time-based morphometric parameters indicates a break point, the threshold size at which metamorphosis occurs. The exact location of these break points is usually approximated using knowledge about the ecology of the organism (E. K. Shea and Vecchione 2002).

Examination of the relative size of key structures was accomplished by plotting each representative structure - eye diameter (ED), maximum mantle width (MW), tentacle length (TL), tentacle club length (TCL), fin length (FL), and fin width (FW) - with Dorsal Mantle Length (DML) as the independent variable (see Figure 3). There was one additional structure measured, the neck length (NL). Shea and Vecchione (2010) mention that measurement of the neck in *Brachioteuthis* was "problematic" because it is very "contractile," resulting in a great deal of variability in the measurements. The necks of *Chiroteuthis calyx* are similarly contractile, and for this reason, the ratio of NL/DML was examined rather than NL alone.

Plots were split based on life stage, as designated by club morphology. The function of the relationship between DML and the size of one of the morphological structures were plotted for each squid life stage in order to visually detect changes in

growth rates and the relative proportions of these structures in a way that would support the assignment of break points. In addition, Analysis of Covariance (ANCOVA) was used to test whether the size of each structure differed between life stages, whether the structure increased or decreased with squid DML, and whether the rate of change (i.e., slopes) in structure size differed between the life stages. ANCOVA analyses used the factors of Life Stage, the covariate of DML, and the interaction between DML and Life Stage. Analyses were performed in JMP v12.

To compare the relative changes in body proportions for each life stage, the length of each structure was standardized to the DML of the squid (e.g, TL/DML), and the mean of that ratio was compared between life stages using a Student's t-test. Some of these ratios were used in an nMDS analysis. These included TL/DML, ED/DML, NL/DML, FL/DML, and FW/DML. The data were normalized, then a distance matrix was created using Manhattan distance. That distance matrix was used to create the nMDS plot, based on the similarities in pairwise distance estimates between samples. If the body proportions change dramatically upon metamorphosis, it was predicted that paralarvae and sub-adults would fall into distinct groups in multivariate space based on these body ratios of multiple morphometric traits.

DIGESTIVE GLAND

The digestive gland is a storage site for ingested compounds, and the pigments it accumulates can provide clues as to what the animal has been eating. Crustaceans are high in astaxanthin, a red pigment, while piscivory tends to produce yellow or greenish digestive glands (Nesis 2002; Almansa *et al*., 2006; Boucher-Rodini and Boucaud-

Camou 2012). The digestive gland color was categorized as red, orange or yellow (Figure 6). It was presumed that the orange color represented an intermediary between red and yellow, as the residence time of the pigments is unknown.

First, the percentage of each color present in each life stage was calculated. Then digestive gland color was plotted against mass to see when the transition in color occurred. It was expected that the majority of paralarvae would have red digestive glands with some orange, while the majority of sub-adults would have orange and yellow digestive glands. The transition from red to orange was expected to happen close to the time of metamorphosis.

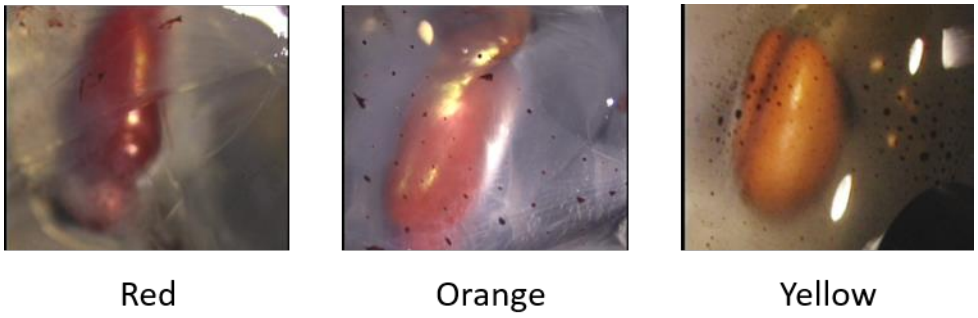


Figure 6: Color change in the digestive gland of *Chiroteuthis calyx*

DEPTH DISTRIBUTION

To determine the depth distribution of these species and their paralarvae, a query was entered into VARS for all *C. calyx* recorded between 1998 and 2013. The search returned 846 entries. These were designated either "paralarvae" or "adults" by presence or absence of the tail, as this is the protocol for VARS Video Lab technicians. The depth distributions of each life stage were compared using a Student's t-test, to look for evidence of ontogenetic descent.

Also of interest was the possible difference in depth variability between the two life stages, as greater variance might indicate vertical migration. However, in an analysis that includes the entire observable size range of the species, it is difficult to tease out variability due to DVM from that of gradual ontogenetic descent. The results of the VARS query included sub-adults and adults much larger and presumably older than those examined in this study. Including only paralarvae and smaller sub-adults in a depth comparison was expected to eliminate variability due to the continuing ontogenetic descent of larger individuals, and make it possible examine the finer scale change in depth and variability surrounding the boundary between paralarvae and sub-adults.

Depth distribution of the animals in this study was examined by plotting depth *vs* mass, and comparing both the mean depth and the variability between life stages. Life stage was again designated based on tail presence or absence, for consistency with the previous depth comparison. 85 specimens total were included in this analysis. This was a combination of fifty four specimens from my collection which had a known depth and a mass value either directly measured or estimated using Equation 3, and 31 VARS individuals for which mass was estimated using Equation 1. It was expected that mean depth would be greater in sub-adults due to ontogenetic descent, and that variability would greater in paralarvae due to a cessation of DVM upon metamorphosis.

RESULTS

MORPHOMETRICS

I examined mid- to late-stage doratopses and early sub-adults of *Chiroteuthis calyx*, the sizes surrounding the period of metamorphosis. The minimum DML was 17

mm, smaller than the minimum recorded size of 20 mm (Roper and Young 1975). The maximum DML in this study was 85 mm, compared to the maximum recorded adult size of 270 mm DML (Nixon and Young 2003). Table 2 summarizes the morphometric results. Of particular note is the size of the tentacle, which increases from a minimum of 17 mm to a maximum of 549 mm. This radical increase indicates that the feeding apparatus will be an important factor in the process of metamorphosis.

Table 2: Summary of morphometric measurements in *Chiroteuthis calyx*. Length is in millimeters, mass is in grams.

	N	Min	Max	Mean	Std Dev
DML	80	17	85	50.33	14.32
MW	39	5	31	16.18	6.79
NL	63	5	29	15.37	5.89
ED	75	1.1	21	7	5.47
TL	49	17	549	146.76	141.77
FL	45	7	34	18.96	5.62
FW	45	4	18	10.04	3.47
A1	39	1	102	32.33	31.11
A2	39	1.5	135	42.09	37.19
A3	39	2.5	157	52.86	43.97
A4	40	10	192	75.63	48.2
Mass	48	0.19	68	14.46	20.26

PARALARVAL STRUCTURES

TENTACLE CLUB DESCRIPTION

In order to map the development of the adult club, each specimen was examined for the presence of a paralarval club, adult suckers along tentacle, adult sucker stalks, a

keel surrounding adult suckers, adult sucker teeth, and terminal photophores (Figure 7). Using these characters, each animal was categorized into one of four developmental stages (Table 3). The first three stages retain the paralarval club, and in the fourth, the paralarval club is resorbed and the terminal photophore forms. At this stage I considered the animal a sub-adult. The distinction between paralarvae and sub-adults for the remainder of the study will be based on these morphological markers, unless otherwise mentioned.

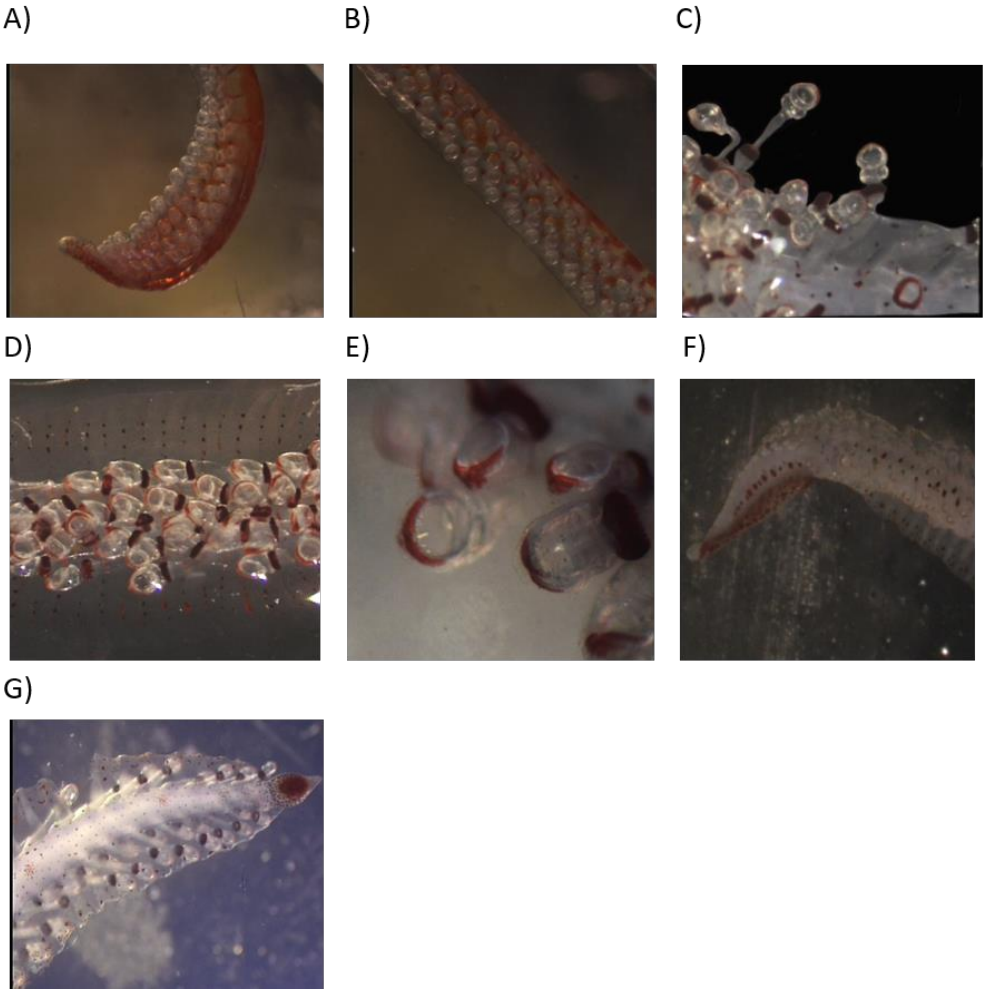


Figure 7: Tentacle club development in *Chiroteuthis calyx*. A) Paralarval club B) Adult suckers in 4 rows along tentacle stalk C) Sucker stalks D) Protective membrane ("keel") E) Sucker teeth F) Adult club formed with paralarval club still present G) Adult club with terminal photophore after paralarval club resorption.

Table 3. Development of the adult tentacle club in *Chiroteuthis calyx*, separated into 4 stages based on the appearance or disappearance of key structures. Stage 4 represents mature adult club morphology.

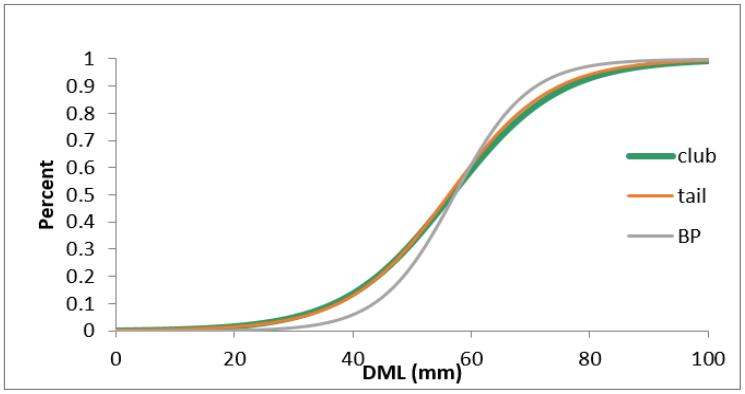
Tentacle Club stages

Stage	1	2	3	4
Suckers forming	Y	Y	Y	N
Stalked suckers	N	Y	Y	Y
Keel present	N	Y	Y	Y
Sucker teeth	N	N	Y	Y
Terminal photophore	N	N	N	Y

PRESENCE AND ABSENCE OF PARALARVAL CHARACTERS

The presence and absence of the three paralarval characters (paralarval club, brachial pillar, tail), were plotted in a logistic regression using both DML and mass as the independent variable, to look for overlap in the point of transition (Figure 8). The inflection point (L_{50} or M_{50}) indicates that 50% of animals have transitioned at that size or body mass into the next life stage. The L_{50} of the three paralarval structures have a range of 0.93 mm, while the M_{50} for all three have a range of 1.34 g. The coefficient of variation (CV) is a measure of the standard deviation relative to the mean. For L_{50} the CV is 0.84%, and for M_{50} the CV is 12.70%. The greater than ten-fold difference in relative variability indicates that DML is a better indicator of life stage, as the loss of all three paralarval structures is highly synchronized with respect to DML.

A)



B)

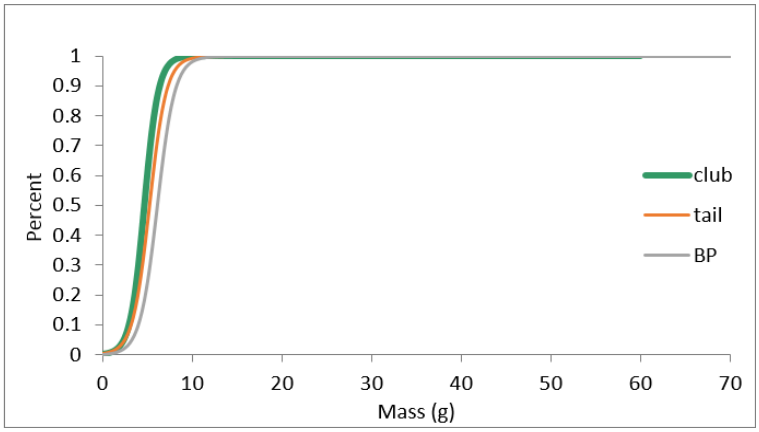


Figure 8: Logistic regression of 3 paralarval structures (paralarval club, tail, brachial pillar) *vs* A) DML (mm) B) Mass (g).

Table 4: Statistics associated with logistic regressions in Figure 8. A) DML (mm) B) Mass (g). **L_{50}** : 50th percentile; LL: lower confidence limit; UL upper confidence limit.

A) DML (mm)

Character	**L_{50}**	**95%LL**	**95%UL**
Club	55.18	50.1	63.5
Brachial Pillar	55.77	50.48	64.98
Tail	56.11	51.34	63.32

B) Mass (g)

Character	**M_{50}**	**95%LL**	**95%UL**
Club	4.71	3.9	5.84
Brachial Pillar	6.05	4.53	10.12
Tail	5.22	4.41	6.62

All 3 paralarval structures (paralarval club, brachial pillar, and tail) are lost at roughly the same time. The paralarvae and sub-adults are grouped distinctly from each other when all three paralarval characters are included in the nMDS analysis (Figure 9). The stress value for an nMDS plot can be an indicator of goodness of fit. Generally, a value between 0.00 and 0.025 is considered "excellent" (Kruskal 1964). The stress value for this test was 0.01, indicating that the distance matrix is an excellent fit for the data.

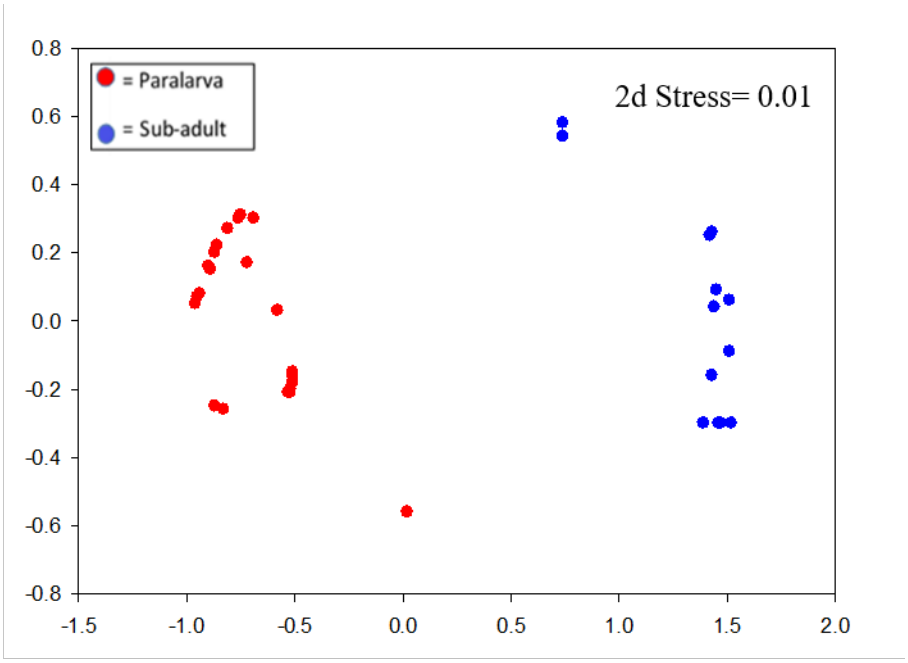


Figure 9: Non-metric multidimensional scaling analysis for presence of absence of paralarval structures in *Chiroteuthis calyx*. Parameters are presence and absence of larval club, tail, and brachial pillar. Distance between points is Manhattan distance.

TAIL LENGTH

Equation 2 was used to approximate the length of n=18 VARS tails for which Equations 1 and 2 were applicable (see Figure 5 and Table 5).

Table 5: Results of tail length (TLL) estimation using Equation 2

	N	Min	Max	Mean	Std Dev
TLL (mm)	18	9.15	231.0	110.73	58.15
TLL (mm) including Eq. 2	36	9.15	279.36	126.68	65.04

Using Equation 3, mass was estimated for 34 squid in my collection, increasing the number of specimens included in further analyses involving mass from N=48 to N=83. A summary of these results is shown in Table 6.

Table 6: Estimation of mass in *Chiroteuthis calyx* using Equation 3. Mass measured in grams.

	N	Min	Max	Mean	Std Dev
Mass (g)	48	0.19	68	14.46	20.26
Mass (g) including Eq 3	83	0.19	68	10.18	16.88

Because tail length appears to be random, I expected there to be very little correlation between tail length and body size. This does appear to be the case, as Figure 10 depicts. A linear regression showed there is no significant trend (Regression: $F_{1,35}=0.26$, $r^2=0.0076$, $p=0.612$) in tail length as a function of mass, with a large variance in tail length of the tail (standard error = 66.15). All collected samples were observed with a jagged or torn appearance at the end of the tail (Figure 10B). This was always the condition regardless of tail length, indicating some mechanical damage rather than gradual resorption.

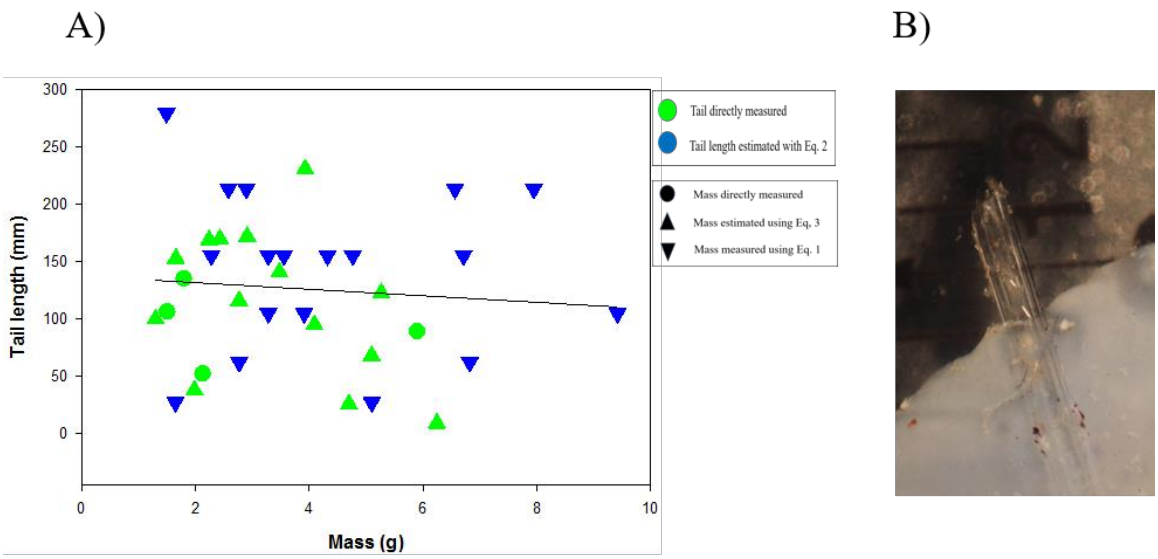


Figure 10: Estimated tail length and appearance of distal ends in *Chiroteuthis calyx*. A) Tail length *vs* mass (n=36) B) Typical distal tail end, with jagged edge.

RELATIVE GROWTH RATES

BODY SHAPE

Mass: The relationship between DML and mass was examined to look for changes in body shape. If mass is being added relatively fast compared to length, it will result in a wider, rounder individual. Figure 11 demonstrates the change in shape of the animal over time. The mass of the paralarvae increases slowly with increases in DML; however, mass rapidly increases with increasing DML in individuals classified sub-adults (based on the presence of the adult club). An Analysis of Covariance (ANCOVA) revealed that the mass after metamorphosis is higher, as is the rate of change in mass as a function of length (ANCOVA; DML: $F_{1, 34}$=132.98, p<0.0001; Life Stage: $F_{1,34}$ =15.63, p=0.0004; DML*Life Stage: $F_{1,34}$ =105.94, p<0.0001).

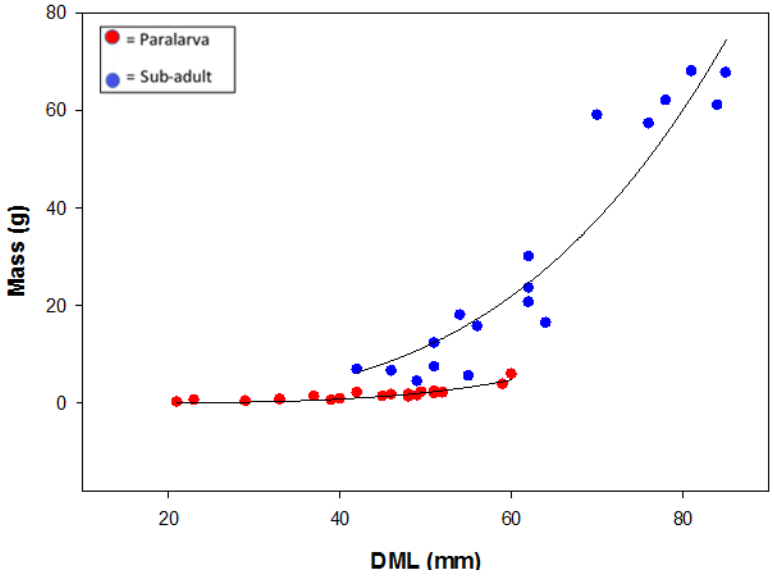


Figure 11: Mass v. Dorsal Mantle Length (DML) in *Chiroteuthis calyx,* power function ($y=a*x^b$). Paralarva: a= 1.26E-7, b= 4.26, r^2=0.84. Sub-adults: a = 1.33E-5, b= 3.50, r^2=0.89

Neck: The ratio of NL/DML is lower in sub-adults than adults. The transition is much more rapid in paralarvae, as the slope is much steeper before metamorphosis as confirmed by the ANCOVA (DML: $F_{1,56}$=32.41, p<0.0001; Life Stage: $F_{1,56}$ =44.63, p<0.0001; DML*Life Stage: $F_{1,56}$ =10.70, p=.0018). Average NL/DML (Figure 12B) is significantly higher in paralarvae (t-test, df=50, t=8.97, p<0.0001). The rapid reduction of the neck greatly reduces the overall length of the animal.

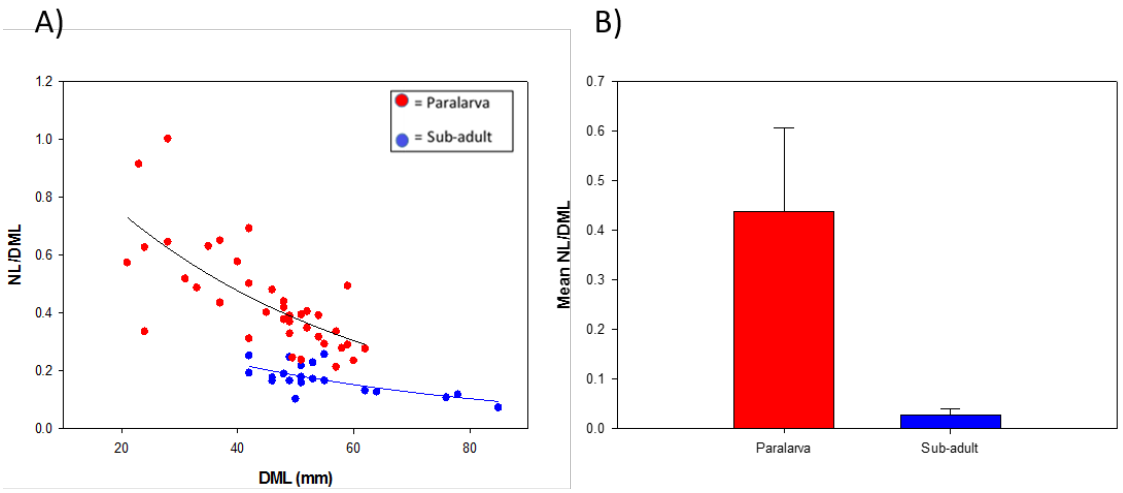


Figure 12: Neck Length/DML in *Chiroteuthis calyx* A) NL/DML *vs* DML exponential decay ($y=ae^{-bx}$). Paralarvae: a=1.17, b= 0.023, r^2= 0.51. Sub-adults: a=0.48, b=0.019, r^2= 0.45. B) Mean NL/DML by life stage. Error bars represent standard error of the mean.

Mantle Width: The mantle width appears to increase upon metamorphosis, creating a larger ratio of width to length in the sub-adults (Figure 13A). After metamorphosis the relative growth of the width compared to DML continues at the same rate as before (ANCOVA; DML: $F_{1,29}$=17.74, p=0.0002; Life Stage: $F_{1,29}$ =17.40, p=0.0003; DML*Life Stage: $F_{1,29}$ =0.64, p=0.4290). The increase of the width upon metamorphosis results in a difference in the mean ratio of MW/DML between paralarvae and sub-adults (Figure 13B). The higher ratio in sub-adults again indicates a more spherical shape (t-test, df=28, t=-4.94, p<0.0001).

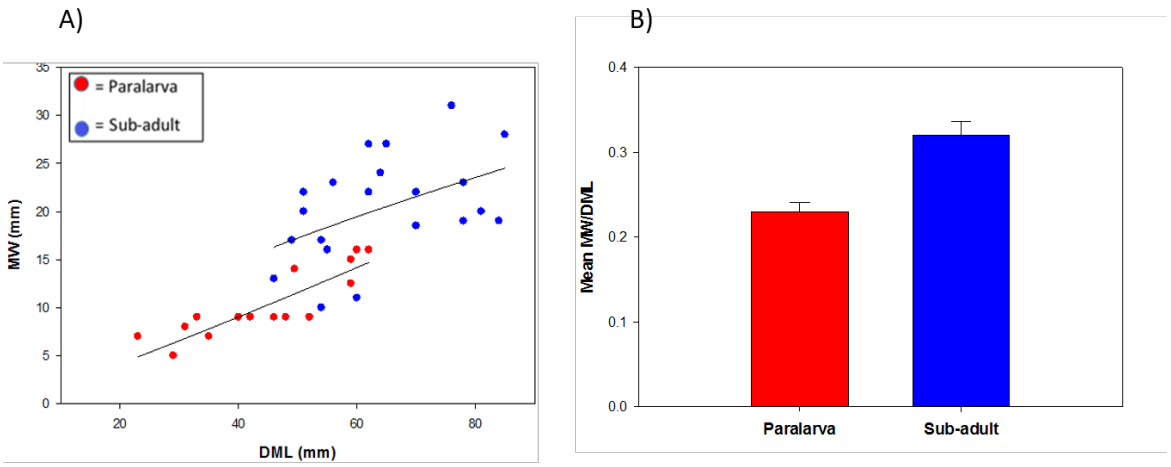


Figure 13: Mantle Width in *Chiroteuthis calyx* A) MW *vs* DML, power functions ($y=ax^b$). Paralarvae a=0.15, b=1.11, r^2 =0.75. Sub-adults: a=1.29, b=0.66, r^2=0.22. B) Mean MW/DML by life stage. Error bars represent standard error of the mean.

Eye diameter: The ratio of the ED to the DML is important in the development of the visual acuity needed to detect and capture faster prey. The ratio of the ED to the DML increases during metamorphosis. In Figure 14A, the size of the eye is much larger in sub-adults than paralarvae of the same length, and the rate of change in ED as a function of DML after metamorphosis is higher (ANCOVA; DML: $F_{1,62}$=167.30, $p<0.0001$; Life Stage: $F_{1,62}$ =165.50, $p<0.0001$; DML*Life Stage: $F_{1,62}$ =24.90, $p<0.0001$). Figure 14B demonstrates the much higher ratio of ED to DML in sub-adults (t-test, df=31, t=-15.02, $p<0.0001$)

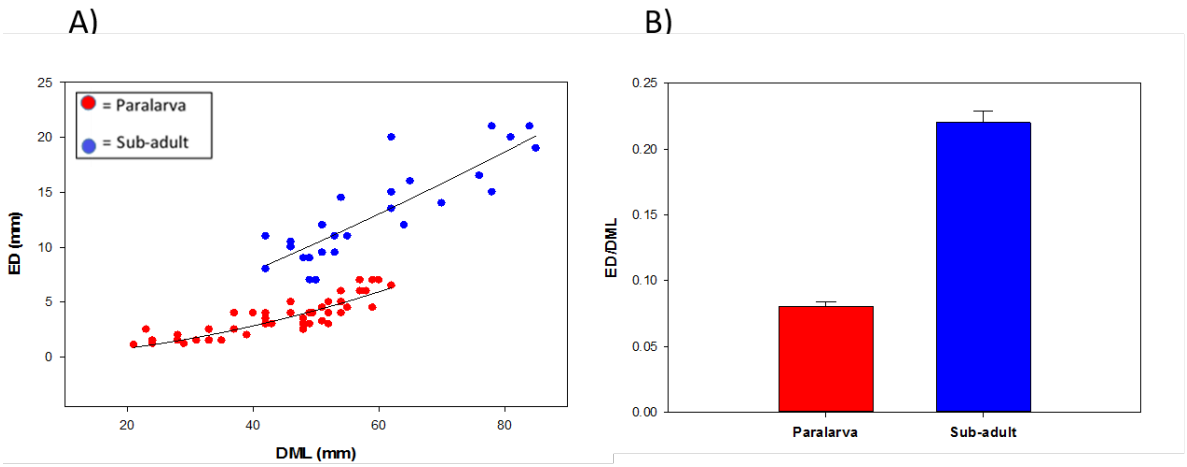


Figure 14: Eye Diameter in *Chiroteuthis calyx* A) ED *vs* DML power functions ($y=a*x^b$). Paralarvae a=.0035, b=1.82, r^2= 0.75. Sub-adults: a= 0.078, b=1.25, r^2= 0.73. B) Mean ED/DML by life stage. Error bars represent standard error of the mean.

FEEDING APPARATUS

Tentacle: the tentacle is the longest appendage and holds the tentacle clubs, which are the main site of prey capture. Tentacle length is indicative of potential prey size. Figure 15A shows an increase in the size of the tentacle as well as the rate of change in tentacle length as a function of DML upon metamorphosis (ANCOVA; DML: $F_{1,37}$=42.60, $p<0.0001$; Life Stage: $F_{1,37}$=78.21, $p<0.0001$; DML*Life Stage: $F_{1,37}$=15.13, $p=0.0004$). Figure 15B demonstrates the much higher ratio of TL to DML in sub-adults, suggesting a larger size of potential prey (t-test, df=17, t=-11.39, $p<0.0001$).

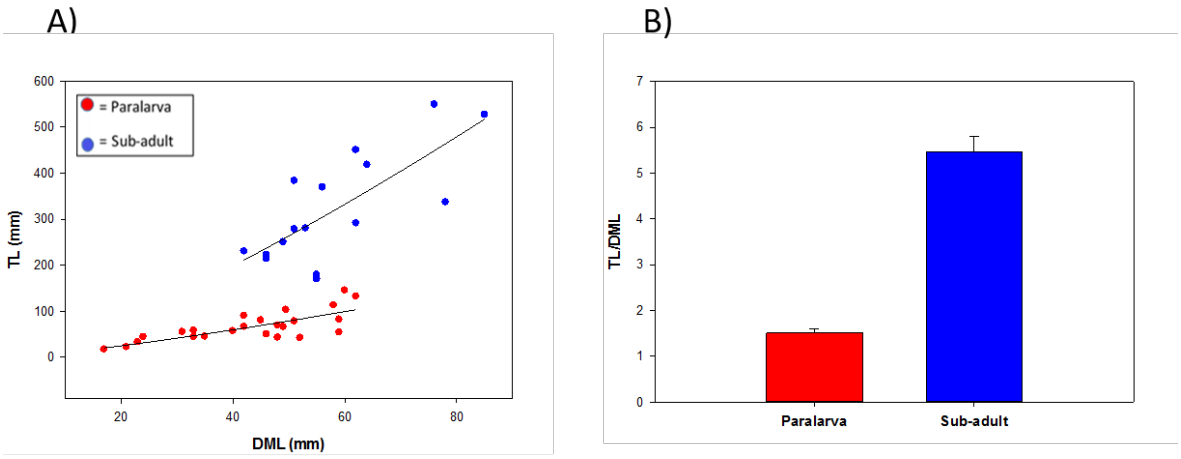


Figure 15: Tentacle length in *Chiroteuthis calyx* A) TL *vs* DML power functions ($y=a*x^b$). Paralarvae: a=0.57 b=1.26 r^2= 0.560. Sub-adults: a=1.82 b=1.27 r^2=0.56. B) Mean TL/DML by life stage. Error bars represent standard error of the mean.

Tentacle club: The paralarval club is smaller than the adult club, and the adult club growth rate is higher (Figure 16A). Measurements of the adult club length were only collected from individuals in which the paralarval club had been resorbed, and so the gradual growth and development of the adult club (recall Figure 7 and Table 3) is not represented in Figure 16A. Because the two tentacle clubs are separate entities, it was not appropriate to do an ANCOVA comparing their relative growth. However, it is still of note that the adult club is much bigger in absolute size, as well as in size relative to the whole tentacle length. The ratio of TCL/TL is significantly different between life stages (Figure 16B), suggesting that the adult club is more involved in the process of prey capture (t-test, df=36, t=-8.66, $p<0.0001$).

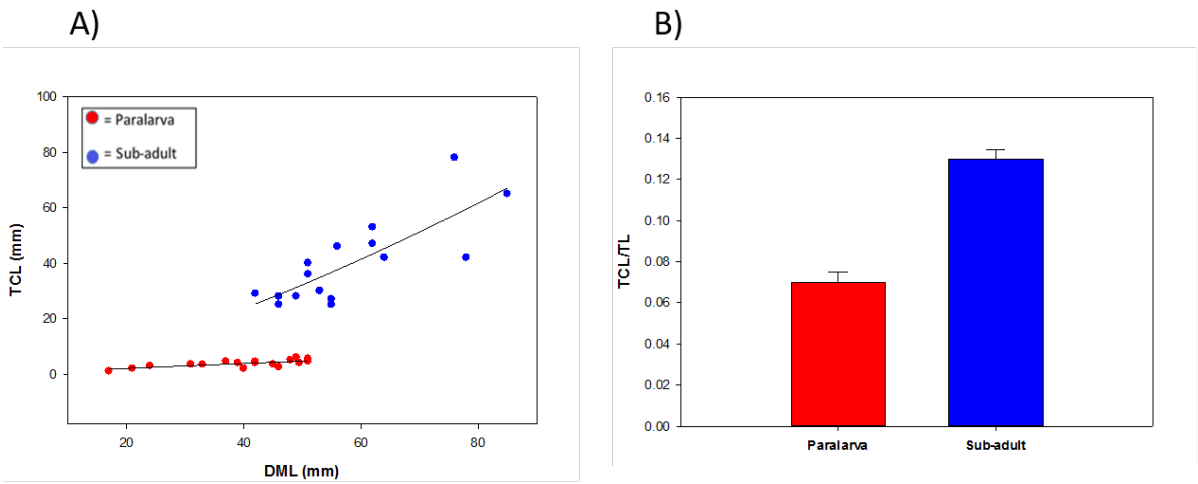


Figure 16: Tentacle club length in *Chiroteuthis calyx* A)TCL *vs* DML.in *Chiroteuthis calyx*, power functions ($y=a*x^b$). Paralarvae: a= 0.13, b= 0.92, r^2=0.53, Sub-adults: a=.14, b= 1.4, r^2=0.80. B) Mean TCL/TL by life stage. Error bars represent standard error of the mean.

Beak: URL was analyzed as an indicator of bite size, and was expected to increase upon metamorphosis. The size of the upper rostrum is indeed significantly greater in sub-adults, and the growth rate of this structure relative to DML is elevated post metamorphosis (ANCOVA; DML: $F_{1,25}$=21.73, $p<0.0001$; Life Stage: $F_{1,25}$ =42.36, $p<0.0001$; DML*Life Stage: $F_{1,25}$ =11.13, p=0.0027). Figure 17B shows that the relative size of the URL is much higher in sub-adults (t-test, df =12, t=-10.66, $p<0.0001$).

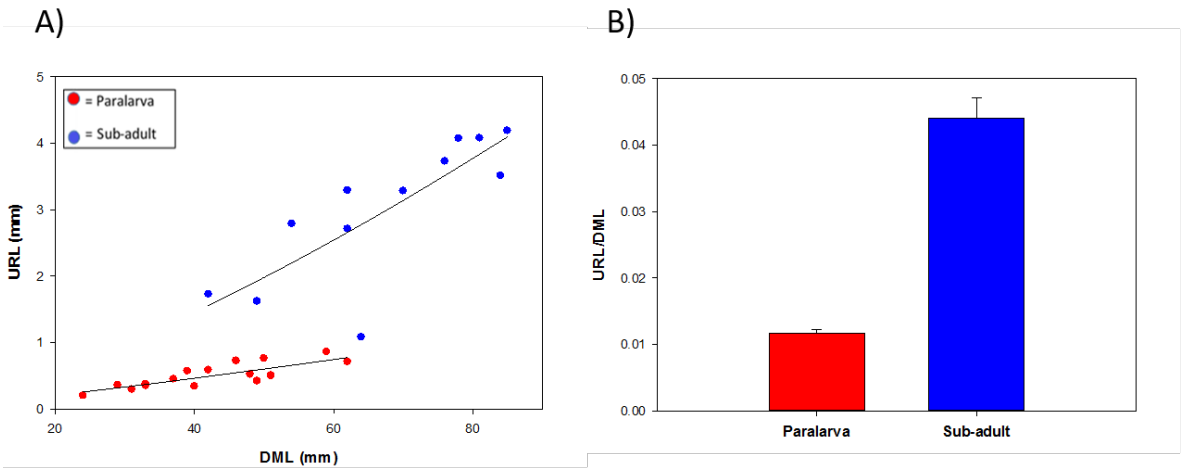


Figure 17: Upper rostral length in *Chiroteuthis calyx* A) URL *vs* DML power functions ($y=a*x^b$). Paralarvae: a=0.0063, b= 1.17 r^2= 0.66. Sub-adults: a=0.0093, b= 1.37, r^2= 0.64. B) Mean URL/DML by life stage. Error bars represent standard error of the mean.

FINS

The paralarvae and sub-adults both use fin swimming as their main method of locomotion. As the adults are neutrally buoyant ambush predators, it was expected that the fins would remain relatively the same size and shape.

Fin length: Fin length does not change in size or growth rate with life stage, though it increases with DML in Figure 18A (ANCOVA; DML: $F_{1,35}$=145.57, $p<0.0001$; Life Stage: $F_{1,35}$ =3.71, p=0.0623; DML*Life Stage: $F_{1,35}$ =1.89, p=0.1778). Mean FL/DML does not change (Figure 18B) so the ratio is stable throughout the transition (t-test, df= 32, t=-0.30, p= 0.766).

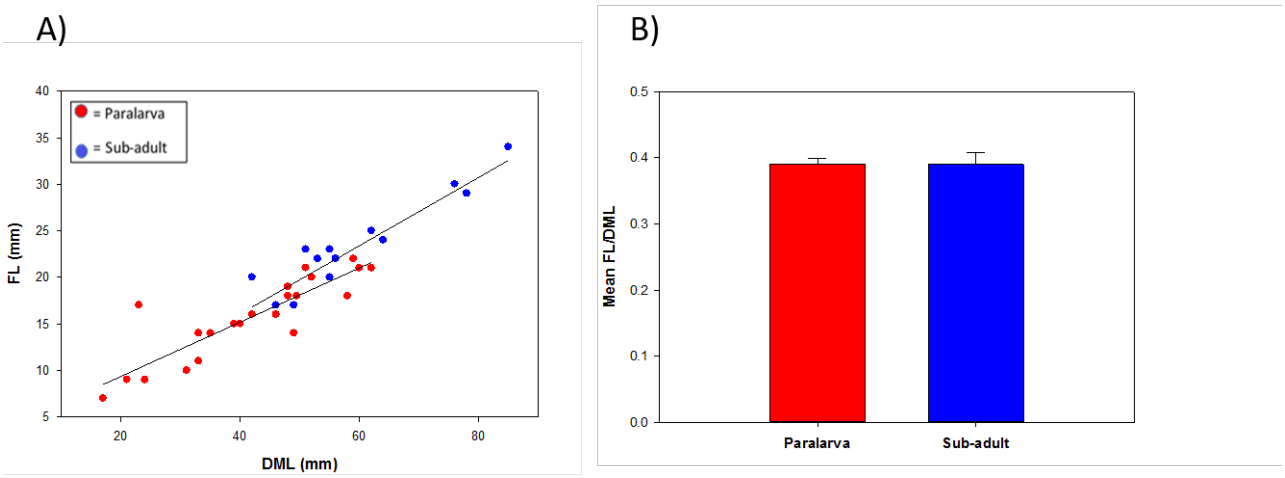


Figure 18: Fin length in *Chiroteuthis calyx* A) FL *vs* DML linear regressions (y=a+bx). Paralarvae: r^2= 0.78. Sub-adults: r^2=0.85. B) Mean FL/DML by life stage. Error bars represent standard error of the mean.

Fin width: Fin width shows an increase in size based on life stage in Figure 19A (ANCOVA; DML: $F_{1,35}$=75.54, p<0.0001; Life Stage: $F_{1,35}$ =6.77, p=0.0135; DML*Life Stage: $F_{1,35}$ =0.11, p=0.7416). Mean FW/DML however does not change, as shown in Figure 19B (t-test, df=37, t=-0.96, p=0.344).

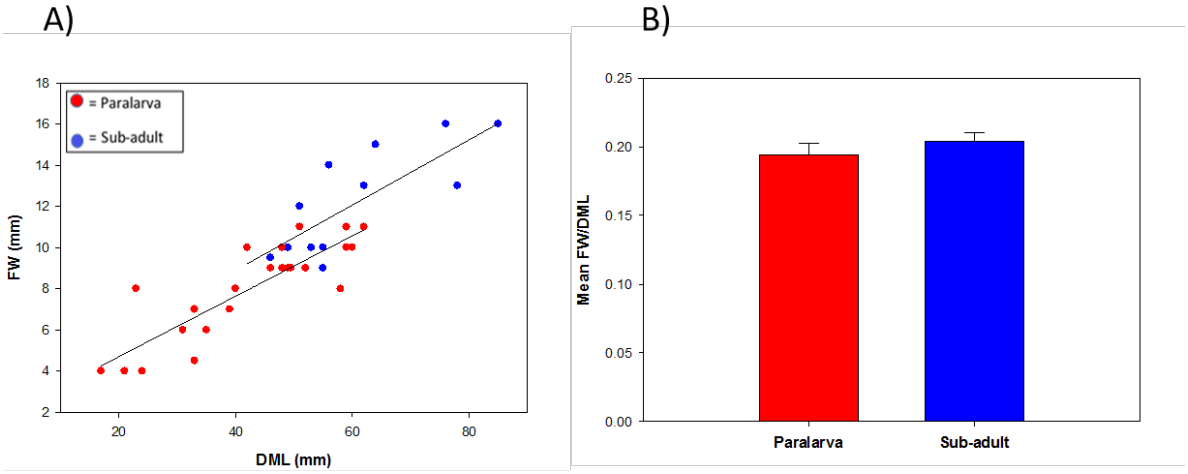


Figure 19: Fin width in *Chiroteuthis calyx* A) Fin width *vs* DML linear regressions (y=a+bx). Paralarvae: r^2= 0.73, Sub-adults: r^2= 0.65. B) Mean FW/DML by life stage. Error bars represent standard error of the mean.

Fin shape: The ratio of FL/FW is roughly 2:1 in both life stages, as shown in Figure 20, which reflects that the fins remain roughly semi- circular before and after metamorphosis (t-test, df=30, t=0.86, p=0.3977).

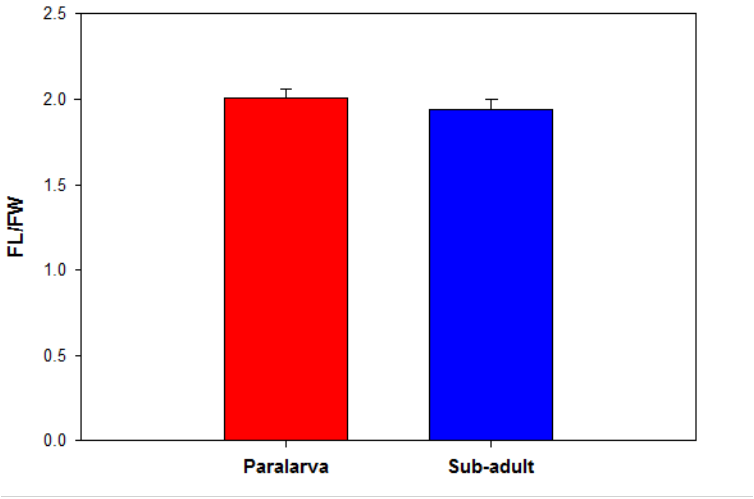


Figure 20: Fin shape in *Chiroteuthis calyx*. Mean FL/FW by life stage. Error bars represent standard error of the mean.

Relative body proportions: The overall change in body shape was analyzed based on the ratios of ED/DML (Figure 14B), TL/DML (Figure 15B), FL/DML (Figure 18B), FW/DML (Figure 19B), and NL/DML (Figure 12B). ED/DML and TL/DML are larger in sub-adults, NL/DML is larger in paralarvae, and FW/DML and FL/DML are the same. These trends between life stages seem to be consistent, as the nMDS (Figure 21) indicates that the two life stages are distinct in their morphology based on the analysis of multiple shared traits. Again, the stress value for an nMDS plot can be an indicator of goodness of fit. Generally, a value between 0.05 and 0.10 is considered "good" (Kruskal 1964). The stress value for this plot was 0.09, indicating that this plot is a good representation of the relationships among the data points.

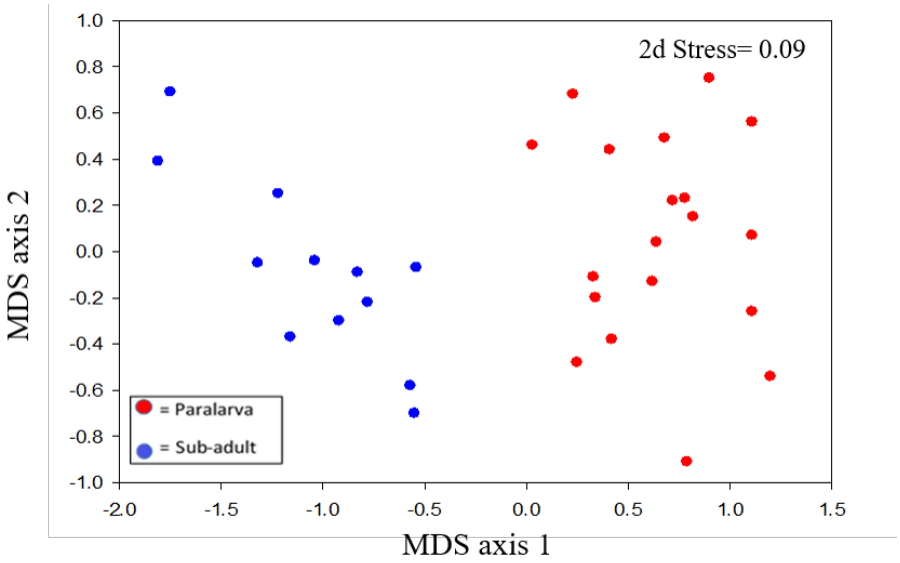


Figure 21: non-metric multidimensional scaling (nMDS) analysis depicting differences in morphometric proportions between the paralarvae and sub-adults of *Chiroteuthis calyx*. Parameters are TL/DML, ED/DML, FL/DML, FW/DML, NL/DML. Distance between points is Manhattan distance.

DIGESTIVE GLAND

39 digestive glands were analyzed (Table 7). Red represents a diet of crustaceans, orange represents a shift from crustaceans to fish, and yellow represents a diet of mainly fish. The majority of paralarval digestive glands were red (83.3%), with 16.7% orange, indicating a diet of mainly crustaceans. The sub-adults had a majority of orange (53.0%), with 35.3% yellow, and a small percentage of red (11.8%) (Figure 22A,B). The sub-adults with red digestive glands were among the smaller individuals, and the sub-adults with yellow digestive glands were the biggest (Figure 22C).

Table 7: Number of digestive glands analyzed, by color

Digestive Gland Color	N
Red	23
Orange	13
Yellow	6

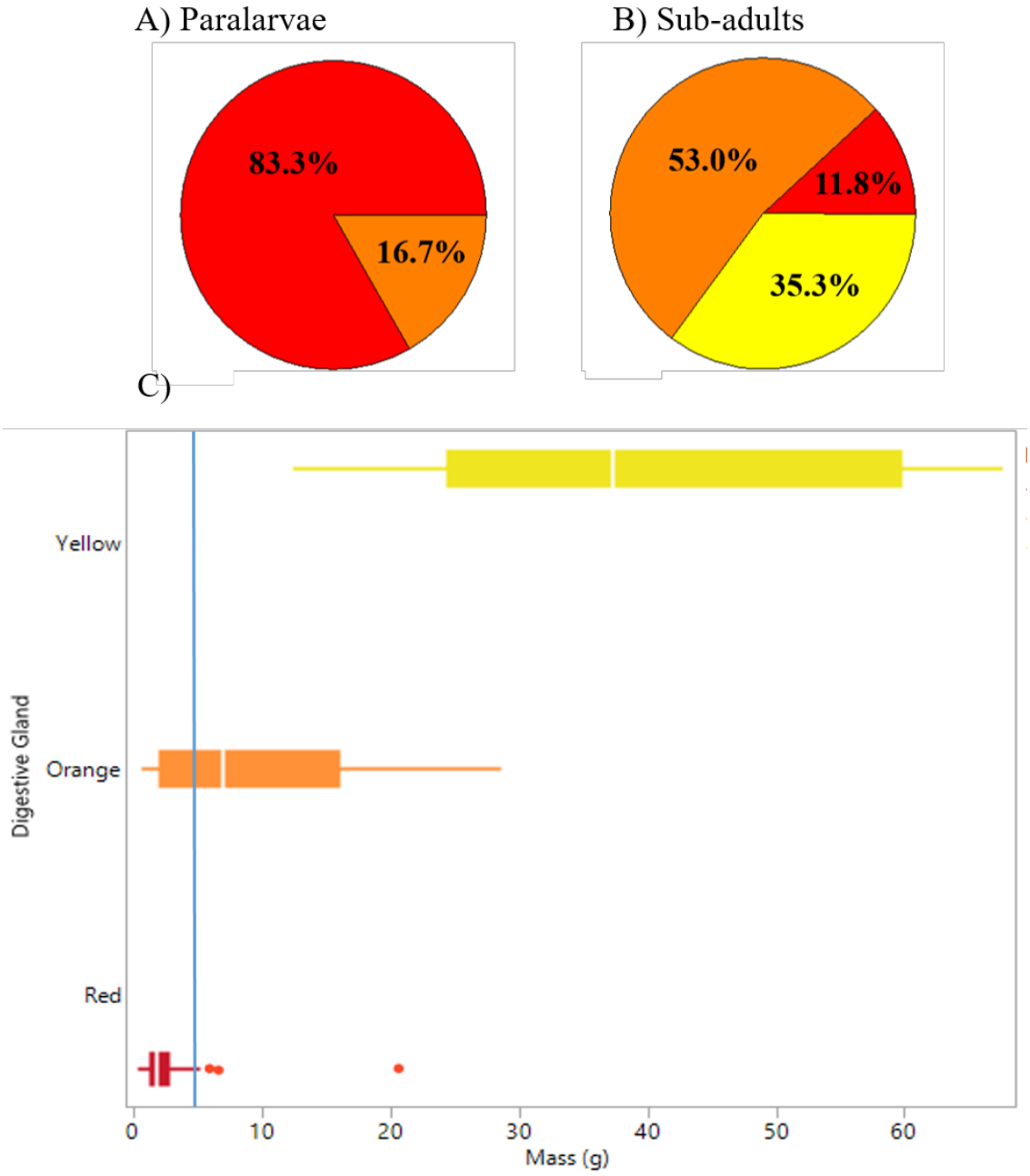


Figure 22: Digestive gland analysis in paralarval and sub-adult *Chiroteuthis calyx*. A) Pie chart showing percentage of each digestive gland color in paralarvae, B) Pie chart showing percentage of each digestive gland color in sub-adults. C) Color *vs* Mass, box plots (white lines in middle of box represent median value, blue line represents mean M_{50} value (Table 4B)

DEPTH

The distributions of *Chiroteuthis calyx* paralarvae and sub-adults/adults from VARS were compared, with the life stage distinction based on tail presence or absence as this was the method used by the VARS technicians. The depth distributions are significantly different between the life stages (t-test, df=833, t=10.9, p<0.0001), though there is a high degree of overlap. The mean depth of paralarvae was 366m (SD= ±124m), while the mean depth of sub-adults and adults was 455m (SD= ±107). The shallowest paralarvae were found close to the surface, where very few sub-adults occurred, while the deepest sub-adults were observed to a depth of 100m, where paralarvae did not occur.

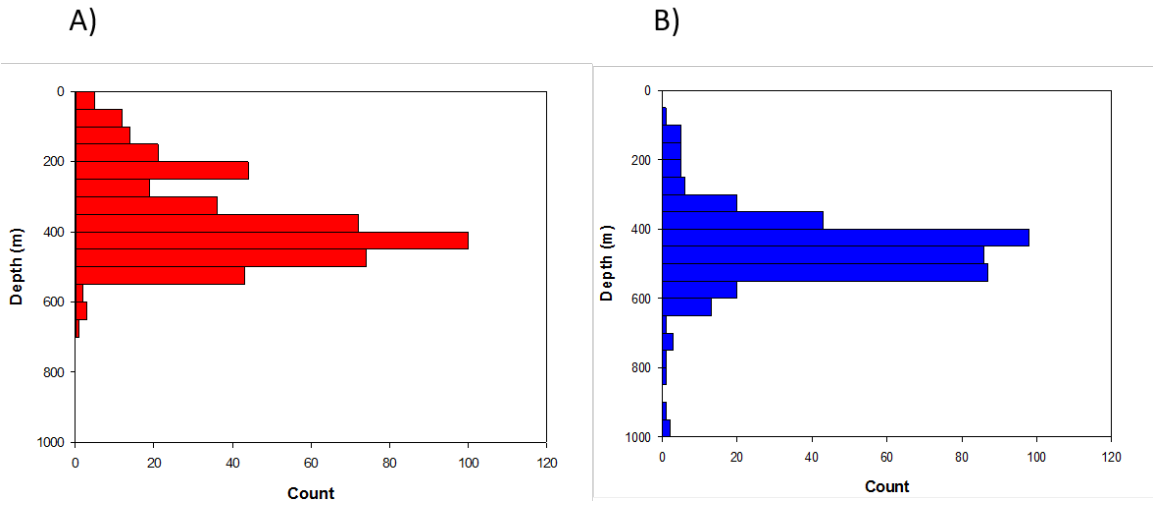


Figure 23: Comparison of vertical depth distributions of paralarval and sub-adult/adult *Chiroteuthis calyx* in all VARS specimens from 1998-2013 (N=846). A) Paralarvae. Mean=366m, SD=124, Kurtosis= -0.11. B) Sub-adults/Adults. Mean=455m, SD=107, Kurtosis=15.5.

The results in Figure 23 support the existence of ontogenetic descent in *Chiroteuthis calyx*. However, variability in depth between the life stages is not equal. The sub/adult and adult standard deviation is slightly lower. The standard deviation in

both life stages may be due to ontogenetic descent alone, but Figure 23B includes a much wider range of animal size/age than 23A. The largest sub-adult in this study was 68 g, with a DML of 68 mm. The largest recorded adult according to the literature is 270 mm, indicating that the animals collected in this study represent a younger age class, surrounding the period of metamorphosis (Nixon and Young 2003).

Including only paralarvae and smaller sub-adults in a depth comparison may eliminate variability due to the ontogenetic descent in the larger sub-adults and adults, and make it possible to examine the finer scale change in depth and variability surrounding the boundary between paralarvae and sub-adults. To this end, depth *vs* mass for animals in this study was plotted (Figure 24). Life stage is again designated based on tail presence or absence, for consistency with the previous analysis.

Mean depths of paralarvae and sub-adults are again significantly different (t-test, df=71, t=4.26, $p<0.0001$). The mean depth of paralarvae in this analysis was 371.6m, (SD = ±138.7m. In sub-adults the mean depth was 466m, (SD = ±71.8). The SD is almost twice as great in paralarvae, indicating that they are much more vertically mobile in the water column. Additionally, the linear regressions show that there is no significant trend downward in either group (Linear regression, y=a+bx. Paralarvae: b=-0.77, r^2=0.0001. Sub-adults: b=-0.40, r^2 =0.012). These results indicates that the change in mean depth from paralarvae and sub-adults is not gradual but abrupt.

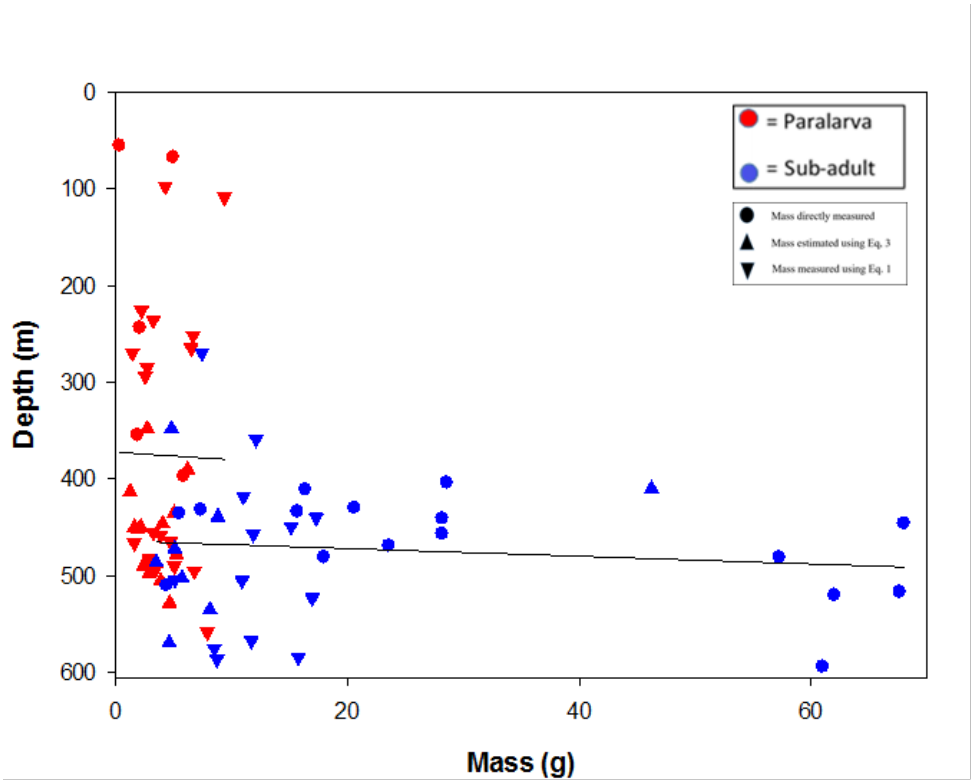


Figure 24: Depth *vs* mass in *Chiroteuthis calyx*. Color of marker designates life stage. Shape of marker represents method of mass determination (direct, Equation 1 or Equation 3).

DISCUSSION

MORPHOLOGICAL CHANGES

The results of this study demonstrate that the process of metamorphosis is rapid in *Chiroteuthis calyx*. The larval characters are lost almost simultaneously, and during this same window the feeding structures show a large increase in size, and the body shape changes as the neck shortens, the mantle widens, and the eyes enlarge, creating an overall thicker silhouette. The fin length and overall shape does not change significantly, but the fin width does increase.

Because all three of the larval characters are lost within a narrow window, we can use any of the three as an indicator of life stage. The tentacle club transition is highly significant, as the method of sub-adult and adult feeding involves the adult tentacle morphology (Robison, 2004).

Vecchione *et al*., (1991) stated that there appears to be "no muscular movement in any structure of the tail;" it is a simple chitinous tube. The pouches along its length are filled with positively buoyant ammonium, and this fluid can be moved into and out of the tail to adjust body position. The expulsion of ammonium from the tail to the body would make the negatively buoyant structure an anchor for downward jetting. When startled, the doratopses orient tail down and quickly jet into darker waters (Vecchione *et al*., 1991; Hunt 1996; Nixon and Young 2003; Burford *et al*., 2014). Arhipkin *et al*., (2015) note that tails are never found in muscular cephalopods, and that they may serve to enhance gliding abilities, facilitating long migrations for these typically weak animals. Thus, the tail could be used by *Chiroteuthis* paralarvae for a more efficient escape. If this is true, then the sub-adults lose the benefit of this gliding ability, and likely are less mobile, perhaps favoring a stay-and-fight strategy aided by their more muscular bodies.

We do not know the tail length of animals smaller than 0.19 g or 17 mm DML, so it is possible that the tail lengths are variable from the time they are formed. However, the ends of the tails are invariably jagged, indicating that the tails start out longer and are broken at least once. Because there is no correlation of tail length with body size, these breaks are likely due to random circumstances. Whatever the reason for the tail shortening, it seems that this process is separate from the final separation of the tail at the insertion. The latter coincides with the loss of the other larval structures, as well as

significant allometric changes in body proportions, prey preference, and possibly depth distribution.

Presumably the tail is the longest when the animals are young, decreasing during the course of the paralarval life. However the high variability in tail length as a function of dorsal mantle length makes tail length a poor indicator of animal size. The ability to approximate tail length using Equation 2 will facilitate size estimation *in situ*, by comparing other structures to the tail. Future studies can use this information to learn more about the ecology of this species.

The higher ratio of TL to DML in the average sub-adult *C. calyx* indicates that their prey is not only larger in absolute terms, but is also larger relative to their body size. The size of the upper rostrum is similarly larger, a common feature in the rapid allometric growth of the feeding apparatus (Vidal 2014). Pelagic biomass exists in peaks and troughs in size, with peaks separated by an order of magnitude (Rodhouse and Piatkowski 1995). An increase in trophic level therefore necessitates that the feeding apparatus grow rapidly to be capable of catching and consuming animals 10 times the size of their previous prey (Boyle and Rodhouse 2005).

The higher rate of mass increase relative to mantle length after metamorphosis is an indicator of a change from elongate and gelatinous, to stocky and muscular (Arkhipkin and Bjørke 1999). The decrease in the neck length and increase in mantle width further contribute to this change in profile. Rapid reduction of the neck has been suggested as a morphological signifier of a change in life stage of *Brachioteuthis* (Elizabeth K. Shea and Vecchione 2010). For the paralarvae, a transparent, gelatinous body renders them almost invisible to predators, and the slender form enables more efficient jet propulsion (Vidal

1994). It is possible that a sleek profile contributes to the jetting behavior of escape mentioned earlier. In larger animals, being dense and muscular will allow them both to wrangle fish and to physically protect themselves. Jetting behavior is likely reduced in sub-adults due to their lack of a tail and wider body shape.

Squid locomotion is a combination of jetting and fin swimming. At rest, fin swimming is the main method of locomotion in both paralarval and sub-adult *C. calyx* (Nixon and Young 2003). In some squid, an increase in the relative size of the fins indicates an increase in their utilization (R. Schroeder 2013; Zeidberg 2004). In this study, the width of the fins increases upon metamorphosis, perhaps to compensate for the simultaneous widening of the mantle. Circular fins are a common characteristic of paralarval cephalopods, and in this case the sub-adult maintains the larval morphology (Elizabeth K. Shea and Vecchione 2010).

With the loss of the neck, brachial pillar and tail, and the widening of the body, the individual becomes less hydrodynamic at the end of the doratopsis stage. The changes in hydrodynamic profile indicate a shift from a mobile to a more sedentary lifestyle (Elizabeth K. Shea and Vecchione 2010). There is a decrease in the locomotive activity and metabolic rate of *C. calyx* with depth related to decreasing light and oxygen availability (Seibel *et al*., 2000; Robison, 2004). Predator-prey interactions occur over much shorter distances when vision is limited, and these more muscular animals may be better suited to a physical defense rather than an escape attempt. A discreet escape is difficult in these darker waters, where any movement is highlighted by a wake of bioluminescent plankton. These conditions favor the sit and wait strategy of ambush

predators, associated with metabolism that is low in activity and highly efficient (Robison, 2004; Partridge, 2012; Vidal, 2014).

Relatively large eyes are common among the Chiroteuthidae, and eye size may be an indicator of depth distribution (Nixon and Young 2003). Deeper dwelling animals tend to have larger eyes relative to their bodies, hence sub-adult *C. calyx* have relatively larger eyes than paralarvae (Partridge 2012; Schmitz *et al*., 2013). Eye size is directly correlated with visual acuity, which is particularly important for catching fast-moving fish in a low light environment (Scharf *et al*., 2000). After metamorphosis the eyes continue to grow at a higher rate relative to mantle length, evidence for their continuing ontogenetic descent.

Shea and Vecchione (2002) used piecewise linear regression to search for break points in their growth curves for 3 species of squid. They find break points in which the difference in the slopes is maximized in several cases. However, these break points occur over a small range of DML. Additionally, they do not discuss differences in overall size before and after the break points; their transitions are smooth. In this study I used morphological information (i.e. the club morphology) to separate the relative growth curves by life stage, and the slopes in most cases were found to be significantly different. However, there is an overlap in the DML at which metamorphosis occurs, and a large gap in size of structures based on life stage.

The smallest sub-adult in my collection had a DML of 42 mm, while the largest paralarva had a DML of 62 mm. This relatively large overlap in DML at metamorphosis can perhaps be explained by varying environmental influence. Size at metamorphosis can vary depending on conditions such as temperature, and seasonal fluctuations in prey

availability (Vidal 1994; Pechenik *et al*., 1998; Hadfield *et al*., 2001; Forsythe 2004; Silva-Dávila *et al*., 2015). The sensitivity of growth rates is especially prominent during the exponential growth of paralarvae.

Also of note is the gap in size based on life stage alone. For example, the longest paralarval TL was 145 mm, while the smallest for the sub-adults was 170 mm. This paralarva was actually longer, at 60 mm in DML, than the sub-adult, at 55 mm in DML, again demonstrating the overlap in DML between life stages. This significant gap in the length of key structures between life stages is also seen in the arm length, eye diameter, and mantle width. The source of energy for this seemingly immediate jump is unclear. It is possible that the paralarvae build up an energy store which is released upon metamorphosis, perhaps due to some external cue, but this occurrence warrants future study.

ECOLOGICAL CHANGES

The change in digestive gland color begins around the time of metamorphosis. Stable isotope analysis has shown an increase in at least one trophic level in *C. calyx* in the range of approximately 50 to 60 mm DML (Anela Choy, pers. comm.). It is likely that the paralarvae feed on small crustaceans, as the stable isotope data found by Choy showed paralarvae feeding on primary consumers. The addition of fish to the diet of sub-adults likely allows for the higher growth rate of structures after metamorphosis, particularly the highly muscular brachial crown (Nixon and Young 2003).

Ontogenetic descent is a known phenomenon in this species, and is supported by the difference in mean distribution between paralarvae and sub-adults/adults (Figure 23). As mentioned, *C. calyx* metabolism and locomotion declines with increasing habitat

depth (Seibel *et al*., 1997; Seibel *et al*., 2000a). If they are less mobile, they are less likely to exhibit DVM. The change from a more to less hydrodynamic shape happens rapidly and in unison, and perhaps the cessation of DVM is coordinated with that change in body shape.

The standard deviation in depth distribution is reduced by half in post-metamorphic sub-adults (Figure 24). Paralarvae are much more mobile vertically in the water column, and so the behavior of DVM is more likely in the paralarvae than in the sub-adults. There is historical evidence of DVM in paralarvae, but similar evidence in sub-adults is lacking (Roper and Young 1975). While I was not able to collect specimens with the ROV's at night, I assumed that migrating specimens collected very early in the morning and late in the evening would be in the process of leaving from or returning to their mean daytime depth.

There is a possible benefit to vertical migration that only the paralarvae may exploit. For their mimicry of *Nanomia* to be effective, the doratopses must occupy a depth range overlapping with that of their model (Speed 1999; Zeidberg 2004). *Nanomia* exhibits diel vertical migration, and so I expect that the doratopses migrate with them, to remain within their densest distribution (Robison *et al*., 1998). This move to shallow water makes the squid more vulnerable as light is more abundant even at night, but the visual mimicry provides protection (Watanabe *et al*., 2006). Vertical migration represents a large expenditure of energy, likely contributing to the higher metabolic activity in paralarvae mentioned earlier. The sub-adults, not relying on mimicry, are not constrained to the distribution of *Nanomia*.

The larval stage is the most vulnerable in all organisms, as animals are small and weak. Thus, larval animals are "often concentrated in nursery areas," where the risk of predation is less and food is abundant (Gibson *et al*., 2002). In the case of *C. calyx*, the distribution of *Nanomia* may provide such a nursery, where they are hidden from predators and have access to abundant zooplankton. The daytime level of *Euphausia pacifica* is between 150-400m in the California current, directly overlapping with the vertical distribution of both *C. calyx* doratopses, and *Nanomia* (Brinton 1967; Robison *et al*., 1998). Cephalopods are generalists, eating whatever is most abundant. It is then likely that the doratopses feed upon the Euphausiids that comprise the majority of *Nanomia*'s diet. Euphausiids are high in carotenoids, consistent with the red digestive gland color of the paralarvae.

It is convenient if the doratopses do share prey items with their model, *Nanomia*. However, long tails are common to all Chiroteuthids, and evidence of mimicry has not been seen in any other members of this family. It is possible that *C. calyx* paralarvae began to consume Euphausiids first, vertically migrating along with their prey, and that mimicry of *Nanomia* evolved secondarily and opportunistically, as their vertical distributions overlapped. When the tail is lost, so is the protection that *Nanomia* provided. At the same time the feeding apparatus rapidly matures into one that allows for the capture of fish, and so the need for vertical migration is eliminated on two fronts.

CONCLUSION

Oceanic squid play a significant role in ocean ecosystems, and given their wide distributions and the large volume of the open ocean, they likely comprise a large percentage of deep sea biomass (Rodhouse and Nigmatullin 1996; Caddy and Rodhouse

1998; Seibel *et al*., 2000a). As predators, the diet of open ocean squid mainly consists of crustaceans, fish and cephalopods, and as prey they are major components in the diets of fishes, seabirds, and marine mammals (Rodhouse and Nigmatullin 1996; Aguilar dos Santos and Haimovici 2001; Piatkowski *et al*,. 2001; Staudinger *et al*., 2013). Squid produce thousands of offspring that grow exponentially, with extremely high growth conversion efficiencies of 20-40% (Boyle and Rodhouse 2005). Their populations are therefore capable of quickly sequestering a large amount of organic material, and the occurrence of ontogenetic descent in many pelagic squid species represents a large transfer of energy to the mesopelagic zone *(Rodhouse and Nigmatullin 1996; Phillips et al*., 2001).

In light of their ecological significance, it is imperative that we gain a greater understanding of their life histories and population dynamics. The derivation of the three equations in this study should assist further research, as particularly Equations 1 and 2 allow for size estimates of length and mass of individual *C. calyx* to be made *in situ* or from VARS footage. This information will allow for further connections to be made between morphological trends and behavioral observations.

The morphological and ecological changes in *Chiroteuthis calyx* seem to be coordinated in a rapid and simultaneous process somewhat unique among cephalopods, and perhaps more closely resembling the radical metamorphosis of other marine invertebrates. Future studies may replicate the methods in this study to determine if the trends seen here are common among other Chiroteuthids. *C. veranyi*, an Atlantic specimen, would be a good target species for such a comparison as it is the most morphologically similar to *C. calyx* (Roper and Young 2013).

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